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MYXOMA AND THE SHOPE FIBROMA. I: THE HISTOLOGY OF MYXOMA.

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IN addition to the lesions recognized and described as typical of myxoma by a number of writers, I have observed other important changes to which no attention has previously been paid. Some of these are most unusual in type, and are probably without parallel in other pathological conditions. From descriptions in the literature, it is clear that the virulence of the strain of myxoma virus used in an investigation, together with other factors, influences the characters of the resulting lesions. In this connection it suffices now to recall the contrasting pictures presented by Findlay (1929), and by Rivers (1930), who obtained better developed tumours with virus stored for long periods (one to two years) in glycerin ; the accentuation of visceral, as opposed to dermal, lesions recorded by Sprunt (1932) in rabbits in an advanced stage of pregnancy ; and the statements of several authors on the effects of frequently passaged virus. Since the following account will serve later as a basis of comparison, it is essential that the course of infection and the histological changes should be described in some detail. Attention will, however, be directed chiefly to aspects of the pathology not previously emphasized, and accepted facts will receive only such mention as is necessary to maintain continuity of narrative.

COURSE OF THE DISEASE.

The strain of virus was one obtained by Sir Charles Martin from Dr. R. E. Shope, and was that used by Rivers ; it was passed at approximately monthly intervals in the testes of rabbits. Owing to interruption of the work it has not been possible to test the effect of more than six months' glycerination on the virus and its lesions ; at the end of this time the latter were perhaps more definitely proliferative than with virus of maximum virulence. In one rabbit, results similar to those described below were obtained with a strain of virus sent to Sir Charles Martin by Dr. H. de Beaurepaire Aragão ; this strain, in Martin's hands (1936) rather less virulent than Shope's virus, has since been passed intratesticularly, and is probably now attaining the virulence

of the other. Most of the rabbits used were adults, but none was pregnant. Virus was introduced intradermally, intratesticularly, or intravenously; except that macroscopically the omentum and mesentery were particularly involved with the second, no constant differences referable to the precise route of inoculation could be determined in the lesions in internal viscera.

After intradermal inoculation of 1 : 2000 and stronger suspensions of virulent testis, a colourless papule was often just visible on the 2nd day, and had by the 3rd day become larger and pinkish; the pink tint faded during the struggling consequent upon anæsthesia, to return within a minute or two. With dilutions a hundred times higher, the first visible lesion appeared on the 4th or 5th day. During the next few days the lesions spread progressively in all dimensions, but especially downwards in the direction aided by gravity; where numerous, the areas ultimately became confluent, rendering the whole back hard and indurated. The edges of the papules were never wall-like, and often merged rather gradually into their surroundings. On excision the skin was highly œdematous, particularly in its deeper layers, as were the subcutaneous tissues; the exuding fluid had a sticky, mucinous character, but, as Stewart points out (1931), such exudates are not confined to myxoma. Small secondary papules appeared on the 4th to 6th days; in one rabbit dying on the 6th day they were absent, and in different cases they varied greatly in number. In one animal used for vaccinial titration a few weeks previously, the distribution of this exanthem was closely related to the sites of the former lesions. By the 6th or 8th day the major lesions and many of the secondary ones showed dull, purplish-red centres not fading on anæsthesia or pressure (hæmorrhagic necrosis). At the same time conjunctivitis and ano-genital œdema, etc., were usually present. Death occurred in from 6 to 9 days, most often in 7 or 8.

After intravenous inoculation of 1 c.c. of 0·1 to 5 p.c. suspensions of virulent testis the course of events was similar. Numerous papules appeared on the shaved back on the 4th to 6th days. In three animals receiving a very virulent virus, sudden death occurred with exanthem on the 4th, 4th and 5th days (*cf.* Sanarelli, 1898; Moses, 1911; Hyde and Gardner, 1933), in the first two despite the fact that before inoculation incisions had been made in the skin of the back to favour localization. In other cases so treated, as might have been expected, myxomatous lesions were more diffuse around incisions of recent date, and more sharply localized with those of 7 to 10 days' standing. Several rabbits inoculated intratesticularly died quite suddenly on the 5th day.

In some cases it was difficult to decide the immediate cause of death. Symptoms in the animals living longest usually conformed to the descriptions of Aragão (1927) and others; the rabbits were quiet and apathetic and refused food for one or two days before death, during which time many manifested deep laboured respiration or paroxysms of rapid shallow breathing. In some the nostrils were unduly moist, and the sounds accompanying the respiratory act, together with gaping of the jaw on inspiration, betokened nasal obstruction. But this obstruction, accepted as the cause of death by Hobbs (1928), did not always appear a sufficient explanation. Sudden death, preceded (when observed) by a few convulsive jerks, occurred in many animals

of which the general condition did not seem very bad. One animal on the 5th day showed extremely rapid and violent breathing imparting a coarse tremor to the whole body; the shaking of its ears was clearly visible from a distance of many feet. On the 6th day breathing was much quieter, except paroxysmally on disturbance. By the 7th day, when the general condition was so bad that the rabbit was killed for histological study, the respiratory disturbance was much less obvious. The precise cause of death in the "septicæmic" type of case was not determined.

The ensuing account is based on examination of 20 rabbits killed in an advanced stage of the disease or when moribund; series of animals examined at earlier stages for special purposes are mentioned later. Where the less common observations were made more than once the number of occasions is indicated.

MACROSCOPIC CHANGES.

Appearances in the skin and subcutaneous tissues need no further comment apart from the statement that, in spite of the supposed susceptibility of myxomatous animals to secondary infection, no sepsis ever occurred at the site of excision of a lesion.

After intradermal inoculation, the regional lymph-nodes were invariably swollen, sometimes pale throughout or containing whitish spherical nodules, sometimes pink or purplish-red with petechial hæmorrhages. Enlargement of other nodes after intradermal or intravenous inoculation was less conspicuous, though microscopical alterations were often present. In most cases the spleen was moderately enlarged, twice to six times the normal, not unduly soft, and showed prominent Malpighian bodies or whitish irregular flecks. In 1 case there was no definite enlargement and the organ was very dark and soft; in 2, moderately enlarged, dark and soft.

In 4 cases the omentum was congested, rolled up and beset with numerous petechiæ. Petechiæ were also present in the wall of the stomach or intestine (4 cases), abdominal fascia, under the peritoneum, in the lungs (3 cases), in the thymus or along the course of the coronary arteries. Larger irregular hæmorrhages occurred in the lungs in 1 case, while, in another, curious "bull's-eye" hæmorrhages were seen. These areas, 1 to 4 or 5 mm. in diameter, consisted of a central, dark red spot up to 2 mm. in diameter and resembling pure blood-clot; the peripheral ring was of brighter and less deep hue. The whole lesion was roughly spherical. This rabbit was among those showing very laboured respiration before death. Such cases showed some emphysema.

In 4 cases the surface of the liver was slightly mottled and the lobular markings obscured; in 3, yellow flecks were present on the surface near the margins of the organ. One case showed marked œdema of the pelvis of one kidney, another what appeared to be an early red infarct in one kidney. A discoloured patch (0·5 × 0·3 cm.) on the surface of an adrenal of 1 case indicated a wedge-shaped area of great congestion and discoloration extending down to the medulla; in several other cases the cortex generally was congested. The tunica vaginalis was involved in 4 cases, the upper pole of the testis in 2. When severely affected, as after direct infection of the organ, the testis was swollen and whitish yellow; the normal markings were largely obscured and hæmorrhages were present. Its translucent appearance and friable consistency then recalled certain neoplasms.

MICROSCOPIC CHANGES.

Skin.

Intradermal inoculation.—The following were the appearances when the animal was moribund on the 7th or 8th day after multiple intradermal inoculations. It will be noted that with this highly virulent testicular virus the lesions resembled more closely those described by Findlay than those by Rivers.

In the dermis, the glairy mucinous fluid appeared in fixed and stained preparations as a typical fibrinous exudate; the strands of fibrin often radiated from a necrotic cell, in many instances

of macrophage type. In some cases no staining was obtained with muci-carmine, in others, the surfaces of the collagen bundles were streaked with pink, and a positive reaction was given also by the protoplasm of certain dead or moribund elements. From observations made in the lymph-glands and spleen (*q.v.*), it seems rather unlikely that the formation of a substance giving the staining reactions of mucin was due solely to mucoid degeneration of the collagen present. Exudate was most abundant in the deeper dermis next to the layer of superficial muscle, where the slight inflammatory changes consequent upon inoculation of normal testicular emulsion were likewise most pronounced, and often in the tissue immediately underlying the epidermis ; it was least abundant in the intermediate zone at the level of, and just below, the bases of hair-follicles. It spread also between the bundles of superficial muscle, and was conspicuous in the loose cellular tissue beneath.

In some places, especially at the margins of a lesion, there was diffuse increase of cells, at most a 2- or 3-fold increase over the number ordinarily present. Few of these had the appearance of elements normal to the skin ; they included myxoma-cells derived from the local connective-tissue cells, large rounded cells of macrophage type, and a variable number of polymorphonuclear leucocytes, many karyorrhectic, which were probably more numerous at an earlier stage. The myxoma-cells have been described by others. Their nuclei were large, and tended to stain heavily at the periphery ; their bodies were stellate and conspicuous, and their characters are well known (Fig. 7). In addition to azurophil granules readily visible in smears stained with Giemsa, their cytoplasm frequently contained a single, feebly or more definitely basophilic spherule some 2 to 3μ in diameter and sometimes giving a weak Feulgen reaction (Fig. 7). In other cells similar bodies occupied an intranuclear position, where they were less definite than the inclusions of Borna disease ; occasionally, however, an oxyphilic spherule rather like one of these type B inclusions (Cowdry, 1934) was seen. Fine fatty granules were present in the cytoplasm. The macrophages exercised their usual function.

Not infrequently the myxoma-cells presented evidence of degeneration. The chromatin of their large vesicular nuclei became condensed to a number of large, deeply-staining blocks, and with the disappearance of its membrane the nucleus became fragmented. This sequence of changes from a nucleus with finely-divided chromatin, through a more vesicular stage in which staining was deeper, to a stage of chromatin fragmentation and ultimate disintegration, was very common in all situations where the peculiar hyperplasia of cells characteristic of the action of the virus was encountered, and the later stages will in future be referred to simply as nuclear degeneration. The fact that in some other situations obviously affected cells show only the nuclear characteristics of myxoma-cells, without the stellate cell-body seen in the dermis, etc., does not necessarily mean that the changes they undergo are not essentially similar to those leading to the formation of typical myxoma-cells ; for example, in the writer's opinion, no distinction, save a minor morphological one, is to be drawn between the type of cell present between the collagen bundles of the dermis, that arising from the adventitia of the vessels, and that responsible for the endothelial thickening to be mentioned later. In the centre of the skin-lesions, and adjacent to the superficial muscle-layer, very few cells were present ; their paucity was due not only to their greater separation by exudate, which, as stated, was most abundant here, but also to the necrosis of many cells. Similar necrosis was prominent beneath the superficial muscles.

The collagen bundles were somewhat swollen, and in places where the exudate was very intense their staining reactions were altered ; with Masson's stain they tended to colour brownish rather than bright green, with phosphotungstic acid hæmatoxylin purplish-blue rather than bright rose, while with Perdrau's method for reticulum some patches were solid black. The elastic fibrils appeared normal.

Remarkable changes in many of the small vessels have, apparently, not been described previously. Rivers (1930) says: "At times the endothelial cells of the capillaries also seem to be involved and are not unlike the large cells seen in other parts of the tumor" ; otherwise these very unusual alterations seem to have escaped attention. The clearest picture was obtained in iron-alum hæmatoxylin, van Gieson, orcein preparations, in which all elements in the vessel walls were effectively displayed.

The distribution of the lesions was often highly irregular in closely adjacent vessels. The venules and capillaries were chiefly affected, and only rarely did the arterioles show changes other than the adventitial proliferation noted below. In affected vessels the epithelium was over-active to a degree never seen by the writer in any other condition ; the cells were proliferating actively and were greatly enlarged in both body and nucleus, while the latter developed in a direction very similar to that taken by the myxoma-cells in the surrounding tissues, and often

showed the same evidences of degeneration. The results varied. Sometimes local bulging into the lumen ensued, in other cases concentric thickening partly or almost completely obstructed the lumen (Fig. 3*b*). In others the surface endothelium was lifted up and the area beneath occupied by typical, stellate myxoma-cells, polymorphonuclear leucocytes, etc. (Fig. 2*a* and *b*). In all these cases the vascular lumen, though narrowed, was still a single structure. But in still other instances the proliferating endothelial cells became more or less stellate and invaded the lumen, where their processes united with those of other similar cells; in this way the lumen was subdivided into a number of separate channels through which, apparently, blood still circulated without thrombosis (Fig. 1*a* to *c*).

In association with the proliferative endothelial change, necrosis often occurred in other elements of the vessel-wall; the collagen was swollen, and the elastic lamina alone retained a normal appearance. In vessels where the endothelium migrated into the lumen instead of remaining as a definite limiting layer, areas of the vessel-wall appeared devoid of this covering, and with the advent of necrosis in other structures only the elastic lamina remained as an effective barrier to the passage of blood into the surrounding tissues; in fact, extravasated red blood-corpuscles were frequently seen around these vessels. The resulting structure was like a sponge through which blood circulated, and which permitted escape of the fluid through the open meshwork of its walls.

From other vessels, particularly the capillaries and arterioles, very similar cells budded off into the surrounding tissue and migrated for some little distance from the vessel-wall, where they took on the appearance of typical myxoma-cells. All these vascular alterations were at a maximum along the line of the superficial muscle, both above and, to a less extent, below it.

The changes described by Rivers in the epidermis (1927) were invariably present and need little further description. To the writer it seems very improbable that they were due to the action of a second virus, as suggested by Findlay. The epithelial layer was thickened by multiplication and swelling of its cells. The surface layer was affected almost as a whole; in the deeper parts of the hair-follicles and the sebaceous glands changes were at an earlier stage, while the growing points of the hairs apparently escaped. Oxyphilic granules first appeared in the cytoplasm, forming a crescent around and near to one side of the nucleus; the cytoplasm then began to liquefy, and as the cell enlarged the nucleus usually came to occupy one pole of the "empty" cell, while at the other a more or less clumped mass of oxyphilic granules mingled with a few basophilic particles which did or did not stain by the Feulgen technique (Fig. 6*c*). A few threads of normally basophilic cytoplasm might at first join the nucleus to the cell periphery. In most cases the nuclei were also swollen, and were either vesicular with a heavily-stained nuclear membrane, or more commonly showed a heavily punctate arrangement of the chromatin. Some, however, were shrunken and pyknotic, irregular or semilunar. Some contained a small amount of weakly acidophilic material less clearly defined than a Borna inclusion. Cell-bridges were no longer obvious, probably owing to swelling of the cells. A few epithelial cells presented the picture of acute necrosis, probably from pressure, since they always occurred at some distance from the basal layer. The surface layers were loose and imperfectly keratinized, but, except in the case of the very severe lesions resulting from intravenous inoculation in animals subjected to incisions in the skin, vesiculation had not occurred; in these whole sheets of the surface epithelium were necrotic, and in other places vesicles were seen.

In some cases, chiefly after intradermal inoculation, the superficial muscle-bundles were in various stages of degeneration, culminating in acute necrosis, when the dead fibre was often buried in a mass of polymorphonuclear leucocytes. The interstitial connective-tissue and vessels showed the changes enumerated above (*cf.* Lipschütz, 1927; Sprunt). The muscle-fibres became homogeneous, with loss of the usual markings; at first more basophilic, they rapidly became strongly acidophilic, while the nuclei were pyknotic and the fibres often vacuolated. The lesions did not spread through dense fascial planes.

In the nerve-fibres of the dermis the cells of the connective-tissue sheath occasionally showed some swelling, and œdema separated the collagen fibrils; for the most part, however, these structures were normal.

Development of lesions.—In two cases the genesis of the lesions was studied by excising pieces of skin at daily intervals after intradermal inoculation; similar excisions from a rabbit inoculated with normal testicular emulsion furnished control material. In all preparations slight acute inflammation was present on the first day. On the second day, on which the first signs of myxomatous reaction were sometimes visible macroscopically, infected tissues differed from the controls in the presence of very scanty fibrinous exudate and congestion of the small vessels, the

endothelium and adventitial cells of which were slightly swollen. Many of the infiltrating leucocytes were now karyorrhectic, and numerous lymphocytes and macrophages were present.

On the third day, lesions in the controls had largely subsided. In the myxoma animals fibrinous exudate was more plentiful. Most of the leucocytes first exuded were degenerate, but around some of the small vessels beneath the epidermis and on the surface of the superficial muscle a fresh leucocytic exudate was abundant. In addition to numerous macrophages there was, in places, slight increase in the number of fibroblasts, but no appearance characteristically myxomatous. The vessels were congested, and the endothelium and pericytes of the venules and capillaries definitely swollen. A fair number of mitoses were present among these cells, and also in foci among the epidermal cells of the deeper layers; the hair had been removed with electric clippers and not by shaving.

On the fourth day there was more inflammatory exudation; blood extravasations were due in part, no doubt, to rupture of the distended capillaries at operation. Leucocytic infiltration and vascular changes along the line of the superficial muscle and elsewhere were much more pronounced, and these changes were spreading downwards between the muscle-bundles. In places enlargement of fibroblasts with definite multiplication was evident at the margins of the papule; in its centre, where fibrinous exudate was greatest, much cellular necrosis had occurred and comparatively few cells survived. Some muscle-bundles showed early degenerative phenomena. In the epidermis specific changes were now definite in many foci.

On the fifth day the changes had further progressed and the first necrosis of muscle was seen; though less widespread and intense than later, all the lesions described above were now represented. A few fibroblasts approximated in size and appearance to typical myxoma-cells, and hence these elements must be considered among the precursors of the abnormal cell type. In the centres of the papules, however, where almost total necrosis may occur, a more frequent source was from the adventitial cells of the small vessels. The nuclei of endothelial cells growing into the lumen of the venules assumed features not unlike those of the myxoma-cells outside.

Secondary skin lesions.—In skin-lesions produced by generalization of virus, the changes at death of the animal were commonly those of an acute inflammation without specific features save for early epithelial involvement; in this connection it must be remembered that in their initial stages the lesions due to intradermal inoculation presented few specific features. Lesions were more conspicuous in the tissues just beneath the epidermis than along the line of the superficial muscle. As previously noted, the epithelial changes had usually progressed further and were more consistently present in the surface epithelium than at the growing points of the hair.

Thus, in the skin an acute inflammatory condition accompanied by necrosis, such as is seen in many virus diseases, is associated with proliferative phenomena of unusual intensity affecting certain mesodermal elements. The multiplying cells undergo a peculiar transformation to be considered later. The magnitude of the proliferation may to some extent be masked by necrosis. In addition to connective-tissue cells, necrosis affects not epithelium alone, as Stewart asserts, but also muscle and fat; the nerve-twigs in the skin are comparatively little affected. Very conspicuous, and of undoubted significance in an understanding of the action of the virus, are remarkable vascular lesions not described by previous observers, and of a type perhaps unique in pathology. At a certain stage granular oxyphilic material is abundant in the cytoplasm of affected epithelial cells.

Lymph-Nodes.

Apart from those studied in animals at an advanced stage of the disease, regional lymph-nodes were removed from rabbits inoculated intradermally 2, 3, 4 and 6 days previously with a 1 : 1000 suspension of testicular virus. In animals near the end of the infection, all lymph-glands did not show the same degree of involvement; in particular the mesenteric glands were usually unaffected, or only slightly affected, at death following intradermal,

intratesticular or intravenous inoculation. Though similar in essentials, the changes in various glands and in different cases were not identical in detail, nor in the regional nodes was the first alteration of structure wholly regular in time. As early as the second day after intradermal inoculation, a few polymorphonuclear leucocytes could often be found in the regional nodes; on the third day definite alterations were usually present. At even an advanced stage, parts of glands might occasionally be relatively normal.

A common early appearance, seen in parts of the periphery of the node between the lymphoid follicles, was a reduction of cells of the lymphocytic series; many of these were obviously degenerating; the nuclei of others were represented by spherical, chromatic globules of variable size. Although often the local reticulum cells were increased in size and number, their increase was not a constant feature at this stage, and there is no doubt that Stewart was right in attributing the lymphocytic outfall to causes other than mere pressure from the proliferating reticulum cells. The lymphoid follicles were, apparently, always affected after the interfollicular zones, and then from the periphery rather than from the centre. In some cases at this stage, polymorphonuclear leucocytes were numerous in the affected regions; deposition of fibrin occurred at times between the cells. The relative intensity of these changes varied from gland to gland; sometimes at an early stage eosinophils were more numerous than polymorphonuclears. Vascular congestion was usual, petechial hæmorrhages not infrequent.

Whatever the earlier changes, proliferation of reticulum cells soon assumed major proportions, spreading inwards along the cords of the gland towards the medulla; these elements increased in size and number as the lymphoid cells progressively disappeared. At the same time, myxomatous changes were often evident in the connective tissue at the hilum and in the walls of the arteries. Nuclear degeneration of the proliferated reticulum cells was of type already described in the skin; individual necroses often followed. Focal and diffuse neutrophil infiltration was conspicuous. Lymphoid follicles were now represented either by focal collections of leucocytes, themselves degenerate, by masses of chromatic globules, or by macrophages filled with such globules; among these, swollen reticulum cells might or might not be seen.

The end-result of the process varied, and could to some extent be correlated with the macroscopic appearances. A swollen pinkish gland was usually found to be in the stage of involvement just described. Those appearing swollen, pearly white and moist (in part or whole) were converted into a more or less uniform mass of stellate myxoma-cells, from which sinuses were absent except at the periphery. More or less polymorphonuclear exudate, diffuse, focal or around the vessels, some persisting lymphocytes, and, in cases in which hæmorrhages had occurred, phagocytes filled with altered blood-pigment, mingled with the stellate myxoma-cells; gradually, however, these other elements tended to be eliminated.

Between the cells was frequently deposited a fibrillar or streaky substance staining green with Masson's method; it did not, however, stain red with van Gieson or become impregnated by silver methods for reticulum fibres. Muci-carmine imparted to this substance a brilliant pink colour, though it cannot be proved that this reaction indicated a true chemical affinity to mucin. The point which deserves emphasis is that this substance cannot here be a product of degeneration of collagen (or reticulum) as might be the case in the skin, for it is far more abundant than are the scanty collagen or reticulum fibrils of the lymph-glands. It seems that it must be accepted as a degeneration product derived from dead cells or, perhaps more probably, as the result of secretory activity on the part of the myxoma-cells, which as abnormal elements of the connective-tissue series may elaborate a product having some relation to collagen. In support of this latter assumption, it may be mentioned that while in most cases there was no appreciable formation of collagen or reticulum fibrils, in one gland the most lively deposition of collagen occurred in close association with the myxoma-cells and walls of the capillaries.

Vascular changes recalled those already described in the skin; many of the capillaries and venules were lined by a prominent endothelium, which often projected in papilliform processes into the lumen, or when less compactly arranged subdivided the lumen into many small channels. Apparently analogous were the obliteration of the central lymph sinuses, and the increased number of swollen reticulum cells bridging in places the dilated peripheral sinuses of the node.

In some glands a few giant-cells resembling, but smaller than, those in tuberculosis were present; as similar cells and groups of epithelioid cells were occasionally found in otherwise normal glands,

this observation probably has no significance. The myxomatous process had often spread through the capsule to involve the surrounding fat and vessels.

In cases where, to the naked eye, the glands were deep red in colour, necrosis predominated, and the proliferation of reticulum cells failed to follow and compensate for the destruction of tissue. The myxoma-cells were widely spaced in an open lacework, and the intervening areas consisted of dilated spaces filled with blood which had escaped from unsupported vessels more severely damaged than those described above. The centres of such glands sometimes seemed to be composed almost wholly of blood.

Similar changes were encountered in lymphoid tissue scattered in a number of situations, such as the walls of the bronchi, the thymus and elsewhere.

Affected lymph-glands show, therefore, an acute inflammatory and destructive condition accompanied by progressive increase of reticular elements ; these undergo myxomatous transformation, replace the degenerating lymphocytes, and perhaps elaborate an abnormal product staining with muci-carmine. Changes in the vessels and lymph-sinuses are comparable with those in similar structures in the skin. The macroscopic and microscopic appearances depend on the balance struck between cellular destruction and cellular proliferation.

Spleen.

In the series of rabbits receiving 1 : 1000 virus intradermally and killed at various periods, the earliest lesions in the spleen were found on the 6th day, but other animals given large doses intravenously or intratesticularly showed advanced changes on the 4th or 5th days.

In the few early cases studied, the disease process began with degeneration and disappearance of lymphocytes, leucocytic and fibrinous exudation, and later enlargement and proliferation of reticulum cells in the splenic pulp immediately surrounding the Malpighian bodies. It spread thence into the remainder of the pulp, and less constantly into the Malpighian corpuscles. The latter were involved more or less uniformly from the periphery, or from only parts of their periphery (Fig. 4*a*) ; areas of normal tissue often persisted until a fairly late stage, and different corpuscles were not always equally affected. The sequence of changes here was essentially the same as in the lymph-glands. There was often more leucocytic exudation in the Malpighian bodies than in the pulp, and at one stage the corpuscles often consisted almost wholly of normal and degenerating leucocytes, with lymphocytic *d bris* and a small number of actively proliferating reticulum cells.

At an advanced stage the macroscopic appearances varied and could be correlated with different histological appearances.

The most common finding was of a spleen twice to four times the normal weight, not very deep red in colour, with numerous prominent white flecks. The latter represented the Malpighian bodies, which now were composed of widely-spaced stellate cells, many showing nuclear degeneration, and but few other elements. Abundant deposition of material staining with muci-carmine and Masson's stain occurred frequently, just as in the lymph-nodes ; in one case it could be stained also by van Gieson's method. The myxoma-cells were usually arranged more or less concentrically around a normal central artery, but sometimes in a number of separate whorls. In the splenic pulp the appearances were quite different. The enlarged reticulum cells did not become widely spaced or stellate, but remained close together, thereby thickening greatly the walls of the sinuses. No deposition of material staining with muci-carmine occurred. Thus the Malpighian bodies stood out in sections as conspicuously clear, relatively cell-free areas in a very cellular pulp (Fig. 4*b*) ; but in neither situation were there more than remnants of the normal cellular structures.

In a few cases, even at an advanced stage, the Malpighian bodies were wholly unaffected, or showed only early involvement at points near their periphery. In such cases no prominent whitish flecks were visible to the naked eye.

In a few animals the victims of fulminating or very severe infections, the spleen was scarcely

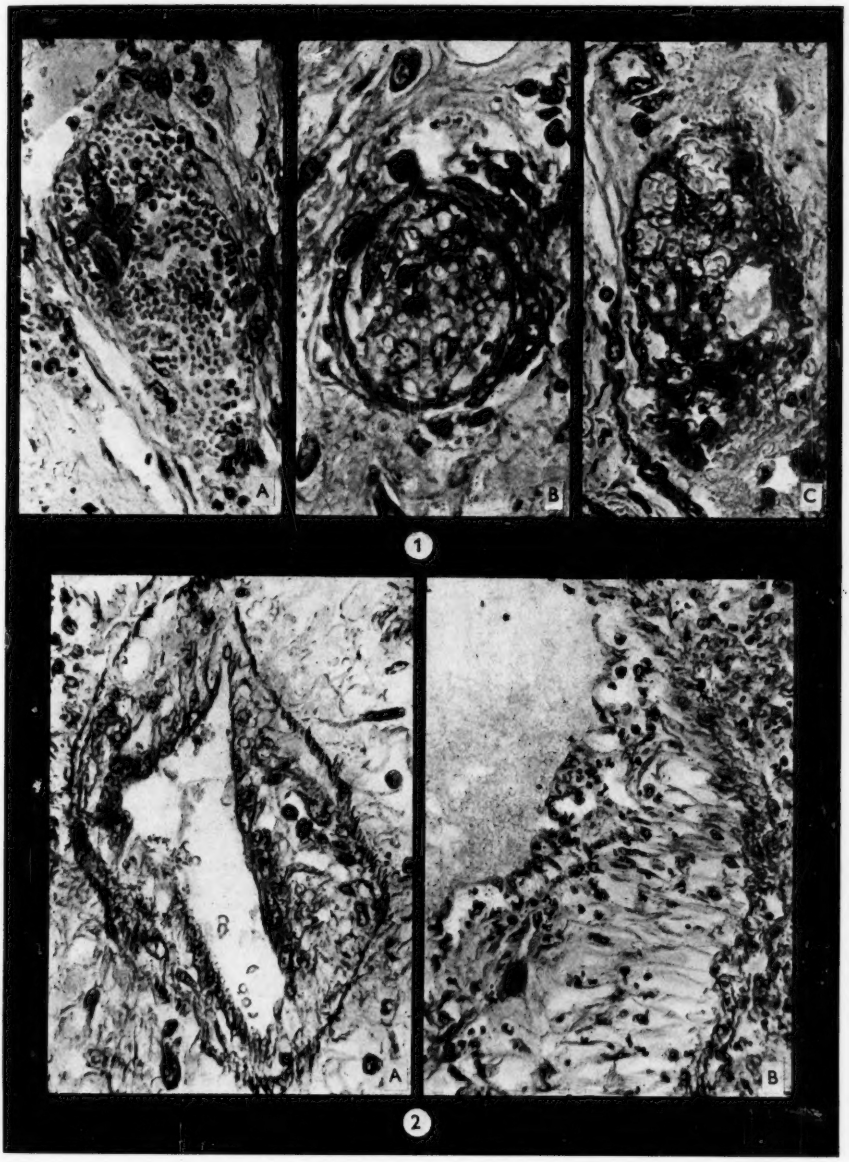

FIG. 1.—*a*, *b*, *c*. Various stages of invasion of vascular lumen by enlarged endothelial cells. In *c* the lumen is subdivided into a number of separate channels through which blood still circulates. Venules in the skin after intradermal inoculation of virus.

FIG. 2.—*a*, *b*. Myxomatous "endarteritis" in venules of the skin (*a*) and lung (*b*). In both cases the elastic lamina has been stained with orcein.

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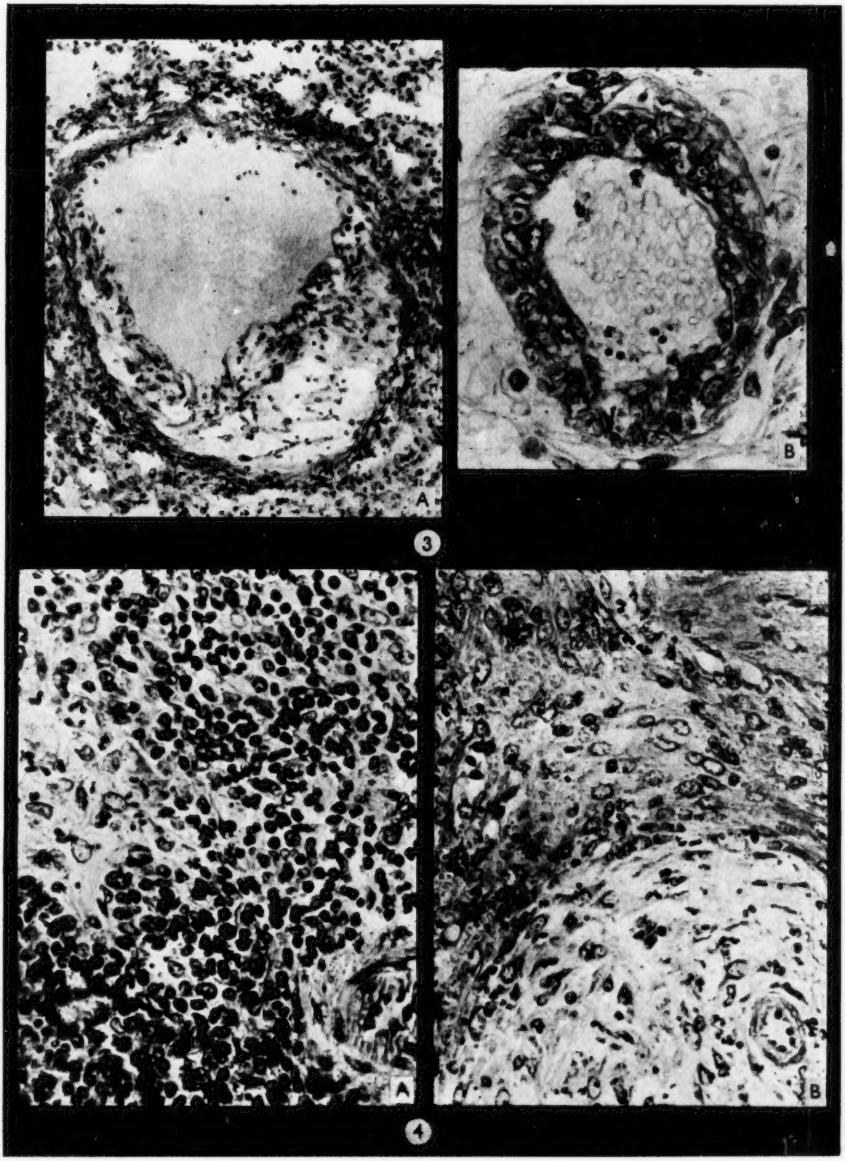

FIG. 3.—*a*. Myxomatous "endarteritis" in a pulmonary vein. *b*. Concentric thickening of a skin venule by hyperplastic endothelial cells.

FIG. 4.—*a*. Early involvement of a Malpighian corpuscle in spleen. The central artery is below and to the right; above and to the left the lymphocytes are being replaced by enlarged reticulum cells and a few polymorphonuclears are present. *b*. A late stage showing the different appearances in the Malpighian corpuscle (below) and the pulp (above) described in the text.

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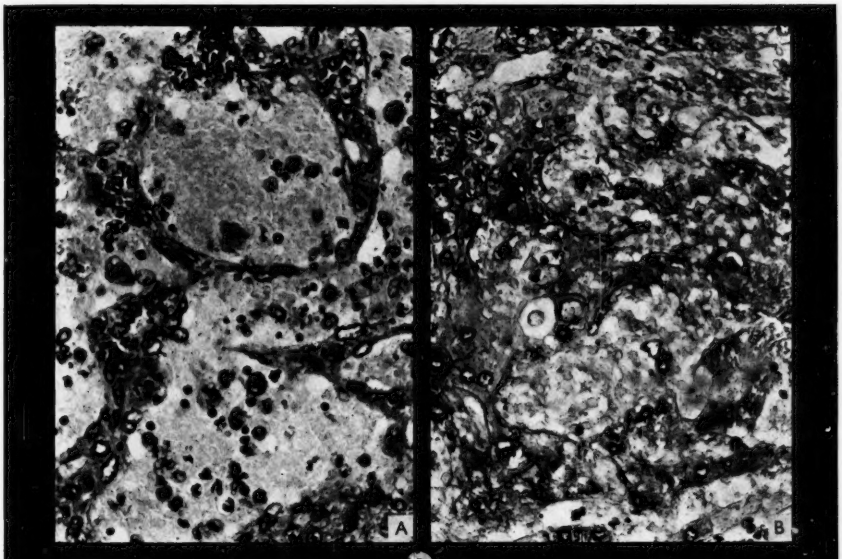

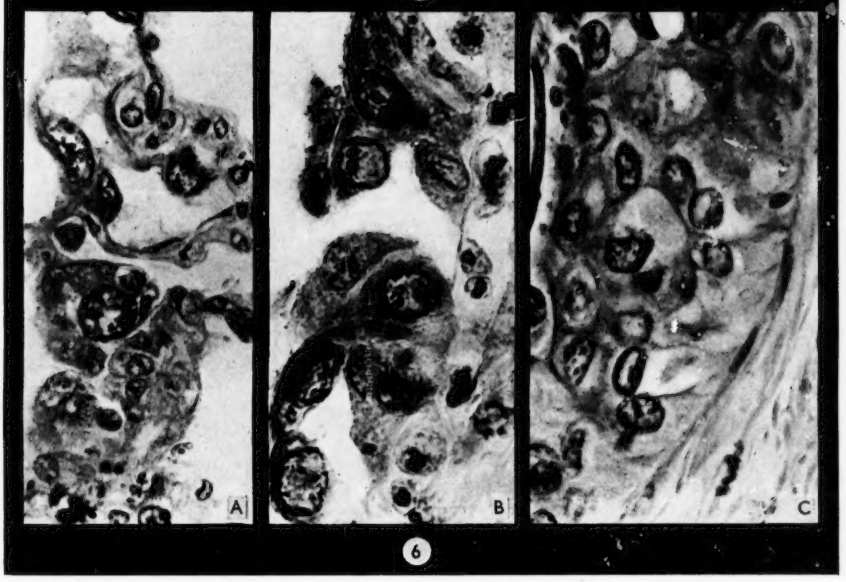

FIG. 5.—*a*. Thin-walled and dilated sinuses in a dark red spleen without prominent whitish flecks; late stage of one type of splenic lesion described in the text. *b*. Great dilatation of hepatic sinuses in midzonal region; marked degeneration of liver-cells.

FIG. 6.—*a*. Enlarged endothelial cells in alveoli of lung. Above they can be seen lining a small vessel. *b*. Hyperplastic mesothelial cells of peritoneum, the nuclei of which are completely filled with granular acidophilic material. *c*. Cytoplasmic inclusions in epidermis.

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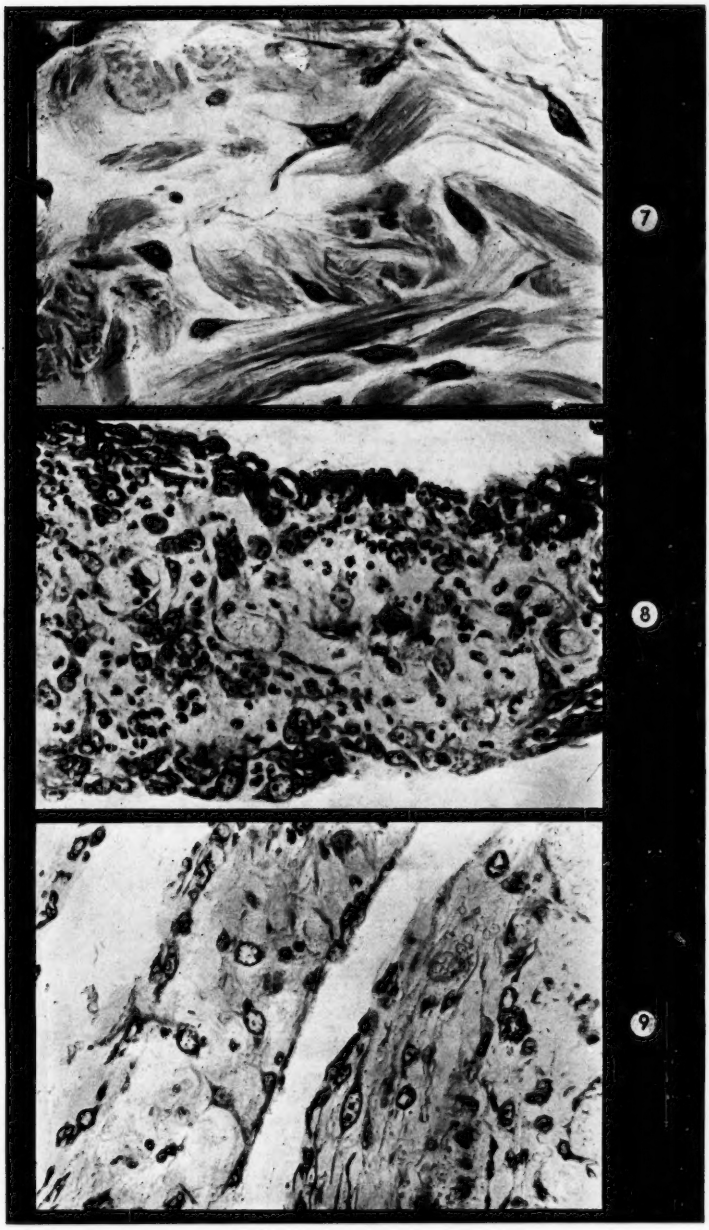

FIG. 7.—Myxoma-cells in skin.

FIGS. 8 and 9.—Myxomatous lesions of omentum. In Fig. 8 the mesothelium is hyperplastic and much inflammatory exudate is present among the myxoma-cells. In Fig. 9 closed spaces lined by swollen mesothelium are formed.

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enlarged and was deep red in colour, with or without whitish flecks. In these cases most areas of the pulp failed to show any great proliferation of reticulum cells. The sinuses were much dilated and stuffed with red blood-corpuscles; their walls, from which most cellular elements other than a few swollen reticulum cells had disappared, were abnormally thin and in places seemed to have given way, with resulting disorganization of structure; often such areas appeared almost as mere masses of blood (Fig. 5*a*).

Lesions in the spleen are essentially similar to those in the lymph-glands; again different appearances result from varying combinations of the destructive and the proliferative processes.

Lungs.

In all but one of 16 animals dead, moribund or estimated to be within a day of death, changes were present in the lungs. Four types of lesion could be distinguished; any might be seen independently of the others, but as the distribution of the first, second and fourth was very erratic, and not more than two or three areas of the lungs were examined, it is possible that their association was more constant than was supposed.

In the first place typical changes occurred in the peribronchial lymphoid tissue; thence they spread into the peribronchial tissue, and along the larger vessels, without, apparently, penetrating their muscular coats. At an advanced stage it was not always possible to affirm categorically that the process had started in a lymphoid nodule, though findings at an earlier stage suggested this. Such lesions were seen in 10 of the 16 rabbits.

Changes in the bronchial epithelium as described by Rivers were found in 6 cases and need no further description. They were not seen in the trachea in 3 cases.

The third change, of very variable intensity, was present in some degree in 11 animals, but in only 5 was it really striking and associated with macroscopic hæmorrhages; it was often, but not invariably, associated with the changes to be described in the sinuses of the liver and adrenals. The condition has received mention only once before, by Sprunt, who stated that in one (pregnant) rabbit there was seen "a generalized infiltration of the interstitial tissue with large mononuclear cells".

In the least affected lungs, a few greatly enlarged nuclei were seen in the alveolar walls; in the most affected the walls were considerably thickened, in foci or diffusely, and many of their constituent cells possessed the large nuclei, and often the abundant protoplasm, of endothelial cells stimulated by the myxoma virus (Fig. 6*a*). Mitoses were numerous, as was nuclear degeneration of the type already described. Occasionally the nuclei contained a pinkish spherule reminiscent of a type B inclusion (Cowdry), very rarely scanty granular acidophilic material; these appearances were very uncommon, and when present the affected nuclei did not show the other changes usually associated with the formation of nuclear inclusions. Many of these cells were phagocytic; their protoplasm was abundant and basophilic. Often they could be seen lining a capillary vessel, but it is not impossible that some arose from extracapillary elements. Sometimes the endothelium of the smaller arterioles and venules participated in this change, and occasionally that of larger vessels. In some cases polymorphonuclear leucocytes were in great excess in the capillaries.

Where this change was most marked, numerous small alveolar hæmorrhages were found. In the animal showing larger "bull's-eye" hæmorrhages, the central areas consisted of blood-clot including scarcely any alveolar walls; those present contained a few enlarged endothelial cells, but were very thin and consisted mainly of degenerate elements. The peripheral zone showed individual alveolar hæmorrhages, and here, as elsewhere in the lung, the alveolar walls were thickened in the manner described.

Two animals dying of fulminating infections showed no enlargement of endothelial cells; on the other hand, many of the nuclei of the alveolar walls were shrunken and pyknotic, and petechial hæmorrhages had occurred in many places. Polymorphonuclear leucocytes were particularly abundant, and there was much perivascular œdema in places.

The fourth morbid appearance was seen in only 2 animals, one inoculated intravenously and

one intratesticularly. Occasional large branches of the pulmonary vein showed great thickening of the intima, concentric or otherwise, by a mass of myxomatous tissue covered with hyperplastic endothelium (Fig. 3*a*). Essentially the same process as that affecting some of the vessels in the skin, the lesion here had spread through the wall of the vessel. Œdema fluid and leucocytes separated the swollen collagen bundles, and in places the elastic laminæ were fragmented ; in the surrounding lung tissue early lesions of the third type were present in the alveolar walls. Elsewhere, however, the latter appearances were wanting, showing that the myxomatous vascular change was not merely an extension of the generalized capillary condition described above.

Though it must obviously be concluded that the third type of lesion, at least when advanced, has a profound influence on respiratory function, no very clear correlation of symptoms with pathological findings was possible. In particular, one rabbit manifesting severe dyspnœa at an early stage and paroxysmal dyspnœa later, when killed on the 7th day showed very little in the lungs save involvement of the peribronchial lymphoid tissue.

In the lungs, again, the importance of changes in the endothelium and reticulo-endothelium is obvious, and again hæmorrhage may be associated with a predominantly degenerative rather than a hyperplastic lesion. Epithelial elements in the bronchi may be affected in a manner similar to those of the skin.

Liver.

Morbid changes were noted in 13 of 17 livers taken at a late stage of the disease ; in 3 of these they were very slight, in the remaining 10 quite definite or pronounced. Once more a number of appearances were seen which might or might not be associated in a given case.

In 5 animals peculiar changes occurred in and around the central lobular veins ; they were less severe than those described by Sprunt in pregnant rabbits. At first sight, in sections stained with methods for the demonstration of collagen (van Gieson and Masson), the veins did not appear grossly abnormal ; but in hæmatoxylin and eosin and in phloxin and methylene-blue preparations the endothelial lining exhibited no trace of nuclear staining, and in many vessels the remainder of the wall, appearing more or less homogeneous, was often completely devoid of nuclear structures. All gradations from the normal could be observed, and in one case sections from one part of the liver showed the change in every vein while other parts were wholly normal. No vessel was thrombosed or blocked in any way. The liver-cells around these veins were much paler and of more "empty" appearance than usual ; they showed no chromophilic material, but only a faint reticulum, and their nuclei were dark and shrunken. In adjacent sinusoids were small groups of lymphocytes and karyorrhectic leucocytes. In 2 of these cases a precisely similar alteration affected the portal veins and surrounding liver-cells. The sinusoids of the liver-lobules lying between affected portal and central veins usually contained perfectly staining erythrocytes, and their Kupffer cells were not necessarily degenerate.

A second change sometimes associated with the preceding, but usually occurring alone (in 7 of 9 cases), was just beginning in 3 cases and well developed in 6. Macroscopically these livers were more or less mottled with yellow-brown areas. The midzonal and sometimes the central parts of the lobules were involved, leaving the portal zones normal. Great dilatation of the sinusoids was accompanied by marked thinning of the cords of liver-cells ; in places, veritable lacunæ 3 to 4 liver-cells in diameter were filled with red corpuscles only once showing conglutination into hyaline masses (Fig. 5*b*). Often numerous polymorphonuclears were present and many Kupffer cells were greatly enlarged, though their nuclei rarely attained the size or showed the coarse clumping of chromatin frequent in endothelial cells of the lungs. Very rarely the nucleus contained a small oxyphilic spherule. Occasionally these cells, or a mass of lymphocytes, appeared to block a sinusoid, but usually there was no apparent obstruction either locally or in the central vein. Most often in these cases the endothelium of the central vein was swollen and projecting into the lumen ; many of the enlarged Kupffer cells were passing out into the vein. Side by side with the hyperplastic Kupffer cells were others of normal size showing degenerative phenomena in their nuclei.

Liver-cells in these areas often stained more deeply with eosin, and owing to less regular

granularity of their protoplasm combined with vacuolation possessed a " ragged " appearance. A few were enlarged, and their bodies filled with granules very like those in the epidermis but not strongly acidophilic. The nuclei, often duplicated, suffered the same kind of degeneration as those in the skin or, alternatively, showed karyolysis or pyknosis. In 2 cases numerous cells, singly or in pairs but never in large groups, showed the picture typical of acute necrosis.

In 3 other cases, parts of the liver near the margins presented massive central or midzonal necrosis with very early infiltration of leucocytes and reaction of the Kupffer cells ; here none of the preceding lesions was noted.

Except perhaps for the enlargement of Kupffer cells, none of these changes was characteristically myxomatous ; in one liver, however, early epithelial lesions like those in the bronchi were seen in some of the bile-ducts, and in 2 others, one of which was slightly cirrhotic, myxomatous change in the connective tissue of some portal tracts.

In the liver the glandular elements show almost pure degenerative phenomena, while the endothelium is either hyperplastic or degenerate. The necrosis of portal and central veins in the absence of lesions in the intervening sinusoids is difficult to explain except on the basis of direct attack of the virus on these structures.

Adrenals.

One or both organs were examined in 17 cases. In 13 instances no significant lesion was observed ; in a number, small oxyphilic intranuclear masses rather like Cowdry's type B inclusions were seen, but these were found also in controls.

In 3 cases the sinusoids of the cortex were greatly congested. Some of the nuclei of their endothelial cells showed degenerative phenomena ; a few were enlarged, but never to the same extent as those in the lung. The nuclei of a few cortical cells were considerably enlarged and vesicular, with heavy chromatin blocks on the nuclear membrane.

In the case presenting a discoloured area in the cortex of one gland, such changes were much more frequent in both adrenals ; this animal also showed marked changes in the lungs and liver. Microscopically, in the discoloured area, the sinusoids were intensely congested, with many extravasations of blood. Many of the endothelial nuclei were pyknotic, or vesicular with fragmenting chromatin ; a few were considerably enlarged. The glandular cells were more pronouncedly eosinophilic than elsewhere. Their nuclei were often dark and shrunken, but sometimes enlarged, vesicular and hyperchromatic like those mentioned above ; their cytoplasmic contents frequently consisted of granules in an " empty " cell as in epidermal cells, but without the marked oxyphilia of the latter. The cell margins were very indistinct. Many of the cells appeared compressed by the dilated sinusoids. The cells of the zona glomerulosa were but little affected, and the medulla showed only congestion.

Evidently on occasion the adrenals may be affected in a manner essentially similar to the liver.

Testis.

Of 7 animals inoculated intradermally or intravenously, 4 presented histologically normal testicles, 2 early changes in the organs, 1 moderate involvement, and 1 considerable involvement.

The earliest lesion detected was in the interstitial cells, the nuclei of which were somewhat swollen and vesicular ; the chromatin was condensed on the nuclear membrane, where it was arranged in a number of intensely staining blocks. In many areas a light leucocytic exudate, with slight fibrinous exudate and karyorrhexis of many of the leucocytes, was also present. In one animal these changes were confined to the zone immediately surrounding the rete testis ; in another the tunica albuginea, interstitial tissue of the epididymis and fatty tissue at the upper

pole of the testis were more involved than the organ proper. In the latter rabbit early specific changes were present in the epithelial cells of the epididymis, and leucocytes were numerous between them. The whole picture in these animals was that of an acute inflammation with relatively little to indicate a definite myxomatous lesion.

The most affected testis of this series showed, in many areas of the organ and its fibrous capsule, a dense polymorphonuclear exudate with considerable deposition of fibrin. In other places the interstitial cells were greatly swollen, both in body and nucleus, and were assuming the characters of myxoma-cells. A few possessed multiple nuclei. Vascular changes of the type already described and hæmorrhages were frequent. In this animal many of the seminiferous tubules were also affected. A few of the germ-cells had undergone acute necrosis; many, apparently affected whilst in mitosis, were degenerating and desquamating into the lumen. Bizarre protoplasmic masses showing a peripheral ring of pyknotic nuclei were often present. But few spermatozoa could be recognized. The epididymis was the seat of typical epithelial and interstitial lesions of severe grade.

Two rabbits killed on the 4th and 5th days respectively after intratesticular inoculation presented a condition not unlike that just described. Interstitial necrosis and proliferation of myxoma-cells were, perhaps, rather more marked, and tubular degeneration less advanced. The interstitium of the epididymis was typically involved, while in the fatty tissue at its upper pole vascular changes were intense and extensive areas of necrosis and myxomatous transformation obtained.

In the testis myxomatous lesions of the interstitial tissue may be followed by degeneration and destruction of the germinal epithelium. The epididymis may be similarly involved, with specific cytoplasmic change in its epithelial cells.

Kidneys.

The kidneys were examined in 16 rabbits; 9 showed no definite change. In 4 mild degenerative changes in the form of nuclear pyknosis, increased eosinophilia of the cytoplasm and desquamation into the lumen were more or less frequent in the cells lining the terminal parts of the first convoluted tubules and the ascending tubules of Henle; in 1 of these rabbits small interstitial hæmorrhages were seen, and blood was also present in the lumina of some tubules. In 1 rabbit occasional endothelial cells in the glomerular tufts were swollen to a lesser degree than those in the capillaries of the lung. In 1 animal there existed marked œdema of the cellular tissues around the pelvis of the kidney.

Finally, in 1 rabbit inoculated intravenously the left kidney showed what looked like an early red infarct. In this organ the cortex generally was the seat of many small interstitial hæmorrhages, and vascular congestion was everywhere intense. In the wedge-shaped "infarcted" area interstitial and tubular hæmorrhages were much more severe. The tubule cells were very degenerate and the lumina occupied by granular and hyaline casts, red blood-corpuscles, or desquamated epithelial cells and karyorrhectic leucocytes. There was no obviously myxomatous change in the stroma, and no evidence of vascular occlusion was found.

Other Organs.

Heart.—Several animals showed a patchy myocarditis, present also in controls. One rabbit dying from a fulminating infection presented many petechial hæmorrhages along the course of the coronary arteries.

Omentum.—In several animals slight changes were seen in this structure; in 1 dying of a fulminating infection many petechiæ were present. In 2 rabbits inoculated intratesticularly and in 2 other animals the omentum was rolled up, thickened and congested, and in places hæmorrhagic. Microscopically, the mesothelial and other connective-tissue cells were converted into myxoma-cells, in one case packed closely together and mingled with abundant polymorphonuclears (Fig. 8), in the other case more widely spaced and associated with fewer polymorphonuclears, eosinophils and lymphocytes (Fig. 9). Where surfaces covered with mesothelium had come partly into apposition, elongated lymphatic-like spaces lined by hyperplastic cells were produced (Fig. 9); in one case acidophilic granular material occurred in some of the nuclei (Fig. 6*b*). Endothelial and adventitial changes in the vessels resembled those elsewhere.

Small intestine.—In 2 rabbits loops of small intestine were thickened and œdematous and filled with a glairy, mucinous fluid. In both, coccidiosis was severe, and an acute inflammatory condition with bacterial invasion of the submucosa was present.

Ovaries and uterus.—In 5 rabbits these organs were normal.

Brain and spinal cord.—No change was detected in 4 rabbits.

COMMENT.

With certain obvious differences in cytological reaction, the effects of infection of epithelial structures with myxoma virus are not widely different from those in many other virus diseases (*e. g.* B virus infection ; Sabin and Hurst, 1935) ; early proliferation gives place to degeneration and necrosis. Necrosis also occurs among cells of mesodermal origin, sometimes especially in muscle tissue ; in general, however, except in very virulent infections, the less highly differentiated mesodermal structures tend to undergo proliferation and hyperplasia without relation, either temporal or spatial, to reparative needs. Hence the view of several observers that myxoma represents one link in a chain between the common infective diseases and the neoplastic conditions (Rivers, 1930 ; Hyde and Gardner, 1933 ; Andrewes, 1934).

The vascular lesions described in this paper at present appear to be unique* ; they may be seen in some degree in most parts affected by the virus. They are severe in the skin, and often in the lymph-glands, lung, etc. ; the sinuses of the liver and adrenal may show analogous but less pronounced changes. On the whole they are perhaps more frequent in capillaries and venules than on the arterial side of the circulation. In certain situations, *e. g.* the central and portal veins of the liver, endothelial degeneration may be more marked than proliferation, or even the only feature ; the petechial hæmorrhages, which may be almost the only macroscopic finding in fulminating infections, doubtless have this origin. It should be emphasized that though the proliferative vascular lesions must have an influence on the nutrition of the tissues, they cannot be held wholly responsible for the necrosis, which is already definite at an earlier stage.

The so-called myxoma-cells have been derived by different authors from fibroblasts (Hyde and Gardner, etc.) and from clasmatocytes (Stewart, 1931, etc.). Stewart states that the fixed endothelium takes no part in their production. The observations just mentioned leave little doubt, however, that the changes in the vascular endothelium in the skin and elsewhere are essentially the same as those occurring outside the vessels, though the final morphological picture may be modified to some extent by the medium in which the reaction takes place. In support of this view we may recall that in the spleen the end-results in the Malpighian bodies and in the pulp are at first sight quite dissimilar, even though in both situations proliferated reticular elements form the bulk of the cells ultimately present. Again, different intravascular processes and cells may differ individually one from another at least as much as the latter, as a group, do from the extravascular myxoma-cells. Speaking quite generally, therefore, we may state that there is a fundamentally similar response to infection on the part of fibroblasts, adventitial

* I am very grateful for the confirmatory opinion of Prof. H. M. Turnbull, who, in his great experience of the rarer pathological conditions, has seen no lesions comparable with these.

elements and vascular and reticular endothelium, all of which tend to develop along similar lines.

Apart from the great hyperplasia, which may finally end in degeneration, an interesting feature in connection with these cells is the abundant deposition in some situations of a substance staining with muci-carmine. Hyde and Gardner have suggested that this is a product of secretion of the myxoma-cells, and not of mucoid degeneration of existing collagen; the writer's findings in the lymph-glands and spleen also suggest that such may be the case. It is worthy of comment that in one instance the formation of collagen fibrils accompanied that of the mucoid substance—an observation which may have some significance in a consideration of the origin of these cells and their potentialities.

At an appropriate stage, no difficulty was ever experienced in demonstrating the cytoplasmic changes described in epithelial cells in various situations by Rivers (1927, 1930); constituting, as they do, one link in a chain of events beginning with proliferation and ending with degeneration and death of the affected cells, they can possibly be regarded as analogous to, though differing in situation from, the intranuclear inclusions of herpes, pseudorabies and B virus disease. In addition, quite occasionally, oxyphilic material has been seen in the nuclei of typical myxoma-cells, affected endothelial cells, and so on. Most often this has taken the form of indefinite flecks, once of massed oxyphilic granules completely filling the nuclei of peritoneal cells, without leaving a halo between themselves and the nuclear membrane. Very rarely, inclusions simulating the spherules of Borna disease, etc., have been observed, but in the adrenal glands of normal rabbits similar bodies may obtain. In view of the irregularity and infrequency of their occurrence, it is impossible at the moment to attach a definite significance to these appearances. It is hoped later to devote attention to the small cytoplasmic granules of the myxoma-cells.

Further comment on the significance of the above findings will likewise be deferred until certain experimental observations have been published.

SUMMARY.

Many of the observations of previous workers on the histology of myxoma have been confirmed. Certain additional appearances, notably those resulting from great hyperplasia of the vascular endothelium, have been described. The relative preponderance of destructive and proliferative lesions is, no doubt, partly dependent on the virulence of the infecting virus. In general, however, epithelial and some mesodermal structures such as muscle tend to degenerate and die when attacked by the virus, while other mesodermal structures such as vascular and reticular endothelium, histiocytes and fibroblasts tend to undergo considerable proliferation.

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MYXOMA AND THE SHOPE FIBROMA. II: THE EFFECT OF INTRACEREBRAL PASSAGE ON THE MYXOMA VIRUS.

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ALL observers agree upon the fatality of myxomatous infection as seen in the laboratory; less than 1 p.c. of rabbits recover from myxoma unless they have passed through a previous infection with the Shope fibroma virus. In studying the serological relationship between the two viruses, it would be very useful to possess a less virulent strain of myxoma; such a strain has been evolved by a now well-known biological procedure, viz. by serial intracerebral passage. Circumstances have not permitted the use of the modified virus for the purpose originally intended, and this paper deals only with the change in its behaviour—itself a matter of some interest.

EXPERIMENTAL RESULTS.

Of 6 rabbits inoculated intracerebrally with virulent testicular virus, all died of generalized myxoma without localized nervous symptoms within the usual period of 7 to 9 days. The virus was then inoculated in series intracerebrally into rabbits, which were killed usually on the 7th day, occasionally on the 6th or 8th days. In this way it has been maintained for 24 generations. At the time of sacrifice most of the rabbits showed typical conjunctivitis, and many showed generalization in the skin; ano-genital œdema was less common. Owing to slight leakage at operation, the scalp sometimes exhibited localized myxomatous change around the tiny incision. A few rabbits showed no symptoms at this stage, though others at the same passage, allowed to live longer, later developed more or less severe signs. Of 30 animals in the series, 2 were completely paralysed in the posterior extremities, and 2 exhibited some

weakness in the same region ; in the remainder no nervous symptoms were noted. Two attempts to pass the virus intracerebrally in fibroma-immune rabbits failed ; in one the virus was lost between the 2nd and 5th passages, in the second between the 1st and 2nd. Evidently the brain is not as suitable a medium for growth of the virus as the testicle, in which, in fibroma-immune rabbits, Shope (1936) carried the virus without difficulty for 8 passages.

The following data on animals not killed for passage or for histology at early stages of infection leave no doubt that a change in virulence has occurred.

Intracerebral Inoculation of Neurovirus.

Of 9 rabbits inoculated with material from the 8th to 19th passages :

(i) 2 died on the 7th and 8th days respectively.

(ii) 5 showed the symptoms typical of myxoma, except that anogenital œdema was absent ; these were most evident about the 10th to 12th days, and all the rabbits recovered within 3 weeks or so.

(iii) In addition to the above manifestations, 2 rabbits showed almost complete paralysis of the hind limbs and sphincters, but were recovering when killed on the 16th and 21st days respectively.

Recently the virus has possibly acquired greater virulence for the brain (but not for the skin), since of 8 rabbits given material from the 20th to 23rd passages, all save one have died within 8 to 14 days. One of these was completely paralysed in the posterior half of the body.

Intradermal Inoculation of Neurovirus.

Material at the 1st and 2nd brain passages gave rise on intradermal inoculation to typical fatal myxoma. No further intradermal test was made until the 8th passage, when a change in the lesions caused by the virus had taken place ; this is described below.

Inoculated intradermally a 1 to 10 p.c. suspension of the modified virus produced at first a papule which was redder, drier and more sharply demarcated than that with testicular virus ; at this stage it resembled more closely the papule produced by old glycerinated virus, or by testicular virus in a fibroma-immune rabbit. About the 6th day the edge was still sharper than with the ordinary virus, and shortly afterwards it often became definitely raised and abrupt instead of merging rather gradually into the surrounding skin. On the 5th or 6th day central necrosis of the lesion was often more intense and more sharply limited, and there was more vesiculation of the epidermis than with testicular virus. Commonly, on the 8th day, the centre of the lesion was deep purple-red, the edge cream-coloured, and the whole papule far drier, both to casual inspection and on section, than in controls inoculated with the ordinary virus ; the lesion was flatter than that of ordinary myxoma, and far flatter than that of Shope fibroma. Thereafter no extension occurred, and by the 13th to 17th day the lesion was represented by a dry scab which ultimately separated with local sepsis. The characters of the skin lesions, and of the disease generally, produced by the brain of the 23rd passage did not differ appreciably from those caused by the 8th.

About the 6th day a variable degree of generalization took place ;

" tumours " appeared on the eyelids, at the bases of the ears, and on the skin generally, and often underwent central necrosis, but ultimately regressed in the same manner as the primary lesion. Conjunctivitis was present, but anogenital œdema was often inconspicuous. With regression of the lesions, hairless patches were left on the ears.

The death or recovery of animals inoculated by the intradermal route possibly depends to some extent on the number of primary lesions, and later of large septic areas. Of 21 rabbits inoculated each in a single area with brains of the 8th to 23rd passages, 16 recovered; 1 died on the 12th day, probably as a direct result of the infection, and 1 each on the 19th and 21st days from secondary infection. Of 9 inoculated in multiple areas, 2 died on the 12th day, probably from the direct results of the infection, 3 died within 16 to 22 days in a marasmic condition, while 4 recovered.

Peripheral inoculation of neurovirus did not lead to nervous symptoms.

Intratesticular Inoculation of Neurovirus.

This was tried because, as is well known, with many viruses this route of infection favours multiplication and enhances virulence. Of 4 rabbits inoculated into one testis, 3 died within 8 to 12 days; the other recovered after a fairly severe illness. The testis of the first to die was passed intratesticularly through a second rabbit, and material removed from this animal on the 7th day still produced the macroscopic and microscopic lesions characteristic of brain virus. This result indicates that while the change due to cerebral passage is not immediately reversible, the modified virus may easily cause fatal infection when given favourable opportunity for proliferation.

MACROSCOPIC LESIONS.

Naked-eye changes were far from pronounced. After intracerebral inoculation the brains were very moist, with slight greyish exudate at the base and in some of the fissures. The cases becoming paralysed in the hind limbs exhibited extremely congested and hæmorrhagic meninges overlying the lower dorsal and lumbar cord; the cord itself was definitely softer than normal, and in 2 cases showed a wedge-shaped, hæmorrhagic and softened area involving the posterior columns and tips of the posterior horns. After all routes of inoculation, the regional lymphatic glands were much less swollen than with ordinary myxoma virus; they were congested, and sometimes presented petechial hæmorrhages. The spleens were often moderately enlarged, dark in colour, and without prominent white flecks on the cut surface; in rabbits dying late they were sometimes greatly enlarged and very soft. In one animal inoculated intratesticularly, the left adrenal was thrice the normal dimensions, and consisted of a central brownish area about the size of the normal gland, surrounded by a thick greyish, gelatinous " capsule ". The inoculated testis itself was firm in consistency and deep brownish-yellow in colour.

MICROSCOPIC LESIONS.

With serial passage a change occurred in the microscopic picture, necessitating separate description of earlier and later passage lesions. In the case of

visceral lesions, comparison may be made with the account already published (Hurst, 1937) of the changes produced by unmodified virus.

Lesions of the Nervous System following Intracerebral Inoculation.

1st to 4th passages.—The brains and general viscera of these 4 animals killed on the 6th or 7th days presented an identical picture. Though widespread, the cerebral lesions were severe in only two situations : (i) in parts of the meninges and the superficial segments of vessels penetrating the brain from these areas, and (ii) in parts of the walls of the ventricles.

Meningitis was confined mainly to the base of the cerebrum and to the lips and depth of the superior longitudinal fissure. It was specifically myxomatous, inasmuch as the mesothelial cells and adventitial cells of the meningeal vessels were actively multiplying and greatly enlarged, having all the characteristics of " healthy " or degenerating myxoma-cells, or, alternatively, their nuclear characters without a typically stellate body. Such cells occurred singly or in small groups in the general meninges, but in the situations mentioned were very numerous (Fig. 1*a*). Similar changes occurred in the adventitial sheaths of penetrating vessels (Fig. 4). Associated in places with this specific lesion was an abundant polymorphonuclear exudate with much deposition of fibrin.* The subpial glial cells were increased in number and size, and in some few the giant vesicular nuclei with their prominent chromatin blocks bore a very close resemblance to those of degenerating myxoma-cells ; sometimes a few leucocytes were present in the nervous tissues. No staining occurred with muci-carmine.

In parts of the walls of the ventricles the ependymal cells were considerably enlarged, with nuclei resembling those of the subpial glia ; desquamation of ependyma sometimes followed. In other places a broad zone of subependymal glia was in active proliferation, with bodies greatly enlarged and nuclei of the type just described (Fig. 3). The walls of the subependymal vessels sometimes showed great enlargement of their adventitial cells, which, on occasion, appeared to migrate into the nervous tissue and then closely resembled the affected glia. But these appearances were unusual, and there was no doubt that most of the enlarged periventricular elements were glial or ependymal in origin.† Still other parts were normal. A few myxoma-cells occurred in the choroid plexuses, where some leucocytic exudate and occasional acute necrosis of epithelial cells obtained. Some of the epithelial cells possibly contained oxyphilic granules in the cytoplasm, though the fixation and staining of the early cases did not allow a final decision on this point, and no such appearances were seen in later cases.

General perivascular œdema was present in all cases. The nerve-cells everywhere were well preserved. The spinal cords were not examined.

5th and 7th passages.—These animals showed appearances intermediate between the foregoing and those described below.

* The material used for infection was bacteriologically sterile.

† This lesion is, of course, not to be confused with the areas of small, darkly staining glial-cells which occur normally in some situations beneath the ependyma.

10th, 12th, 13th, 15th, 16th, 18th, 19th and 22nd passages.—Allowing for differences in severity and duration (6 to 13 days) of the disease process, the histological picture was now uniform, and differed materially from that at the earlier passages. It had lost its specifically myxomatous character.

Meningitis was severe in the situations mentioned above, and was present also over the brain-stem and cerebellum and to a variable extent down the entire length of the spinal cord. It was now wholly or almost wholly mononuclear, with lymphocytes, and later plasma-cells, as the predominant type of cell. In addition there was variable and often considerable multiplication of meningeal elements, which, though possessing somewhat enlarged, pale and perhaps vesicular nuclei, were much smaller than myxoma-cells, and showed neither the heavily staining and frequently fragmented nuclear chromatin nor the stellate bodies of typical myxoma-cells (Fig. 1*b*). No staining with muci-carmine occurred. After 12 to 13 days new collagen was being laid down by proliferated cells which had assumed the appearance of fibroblasts. Often the endothelial cells of the venules in more severely affected regions were swollen and more numerous than normal, but to no greater extent than occurs in other virus diseases characterized by a direct attack on the meninges. The perivascular infiltrate around the superficial parts of penetrating vessels was composed of cells similar to those in the meninges ; such infiltration was often especially prominent in the lower spinal cord.

The subpial glia in brain and cord was uniformly or patchily enlarged and multiplied, again without the striking nuclear appearances of earlier passages ; occasionally similarly affected glial cells were seen among the cortical nerve-cells, but save in the rarest instances the nerve-cells were perfectly normal in their staining reactions. The choroid plexuses were usually normal, though in the rabbit of the 22nd passage the epithelial cells were almost wholly destroyed, and the stroma densely infiltrated with lymphocytes and other mononuclear elements. In 3 cases the ventricular walls (and central canal of the cord) exhibited ependymal and glial proliferation with some lymphocytic exudate ; no large nuclei were present.

In the 2 cases examined with paralysis of the posterior extremities, lesions in the lower dorsal and lumbar cord (but not in the upper dorsal or cervical regions) were very much more acute and severe. An intense meningitis, most pronounced behind, had resulted in necrosis of meningeal cells, of the vessel walls and of the swarms of invading leucocytes ; in addition much fibrin was present. Thrombosis often affected necrotic veins over and in the posterior columns, and it was this vascular disturbance which was responsible for the partial, hæmorrhagic softening of the posterior columns and parts of the posterior horns. Anteriorly the intensity of the inflammation was less, but in certain posterior nerve-roots and spinal ganglia there existed much interstitial inflammatory exudate of mainly mononuclear composition. Although in the ganglia a number of nerve-cells were acutely necrotic and often buried in leucocytic exudate—the only necrosis of nerve-cells observed in these cases apart from that obviously due to vascular causes—the inflammatory condition appeared out of all proportion to the number of damaged nerve-cells. In one ganglion, necrosis of isolated nerve-cells occurred in the absence of general infiltration.

Lesions of Different Organs following Inoculation of Neurovirus by Various Routes.

Viscera in general.—In the rabbits of the 1st to 4th cerebral passages very early myxomatous changes were found in the spleens, and more advanced lesions in the deep cervical lymph-nodes. Other organs were normal, but it must be remembered that many animals killed on the 6th or 7th day after inoculation by other routes would show no definite changes in most of the viscera.

At later intracerebral passages, as well as after intradermal or intratesticular inoculation of neurovirus, though ample time (7 to 21 days) had in many cases been allowed for the development of any characteristic myxomatous lesions, appearances were very different from those seen in ordinary myxoma. The clinical picture, though somewhat modified, was in all cases undoubtedly typical, but except in the epidermis, there was little or nothing microscopically to suggest the precise nature of the lesions.

Skin.—This was examined 3, 5, 7, 9, 11 and 14 days after intradermal inoculation. At the earlier periods, acute inflammatory phenomena were much more pronounced than with testicular virus, and, from their intensity at the spreading edge of the lesions, it seemed that this accentuation was not wholly due to the greater amount of tissue in the suspension used. Although enlarged connective-tissue cells were present, no cells which would have been accepted as typical of myxoma could be found in the sections. By the 5th day necrosis in the dermis and layer of superficial muscle was already well defined, as were specific changes and vesiculation in the epidermis. By the 7th day the whole centre of the lesion was necrotic down to the layer of superficial muscle ; some vessels here were thrombosed and their walls necrotic. In addition, many muscle-fibres had succumbed and were in various stages of resorption ; the nuclei of others were greatly enlarged. At the margin of the necrotic area on the 7th and 9th days, the epithelium still exhibited vesiculation, formation of inclusions and nuclear multiplication (in this order from the centre) ; by the 14th day specific changes were absent, and a new covering layer was beginning to grow inwards over the necrotic zone. The inclusions just mentioned were the only appearances in the skin at all typical of myxoma as it is usually known. In the dermis bordering on the dead central area, although considerable multiplication of connective-tissue cells had taken place, none had assumed the nuclear or cytoplasmic peculiarities of typical myxoma-cells, and all resembled more closely young fibroblasts. In addition, abundant lymphocytes and macrophages and fewer polymorphonuclears were present around the vessels and elsewhere. Without knowledge of the clinical course of the illness, and of the change occurring in the histological picture in the central nervous system, these mesodermal lesions would never have been considered myxomatous. Less frequently than in unmodified myxoma (Hurst, 1937), the small veins showed considerable proliferation of their endothelium, resulting in great narrowing of their lumina, but again the cells no longer possessed the relatively enormous nuclei and bodies seen in infections with unmodified virus, but were of almost normal size. Fibrinous exudate was scanty, and no staining was obtained with muci-carmine.

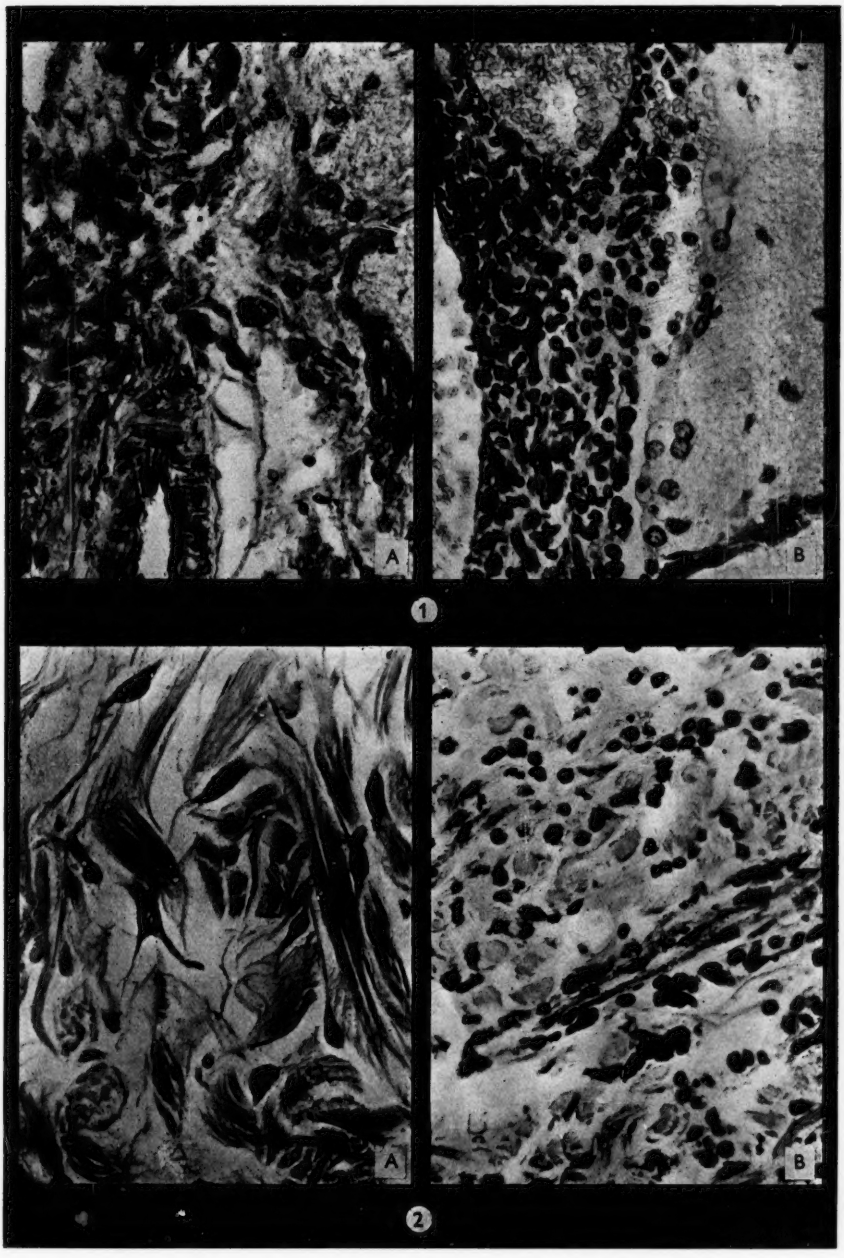

FIG. 1.—*a*, Typical meningeal reaction at second serial intra-cerebral passage of myxoma virus showing large myxoma-cells in meninges. *b*, Typical meningeal reaction at tenth passage showing absence of specific lesions. In both cases the magnification is the same.

FIG. 2.—*a*, Typical myxoma-cells at edge of a skin-lesion produced by intradermal inoculation of testicular virus—8th day. *b*, Typical skin reaction at edge of a lesion due to intradermal inoculation of neuromyxoma—9th day. In both cases the magnification is the same.

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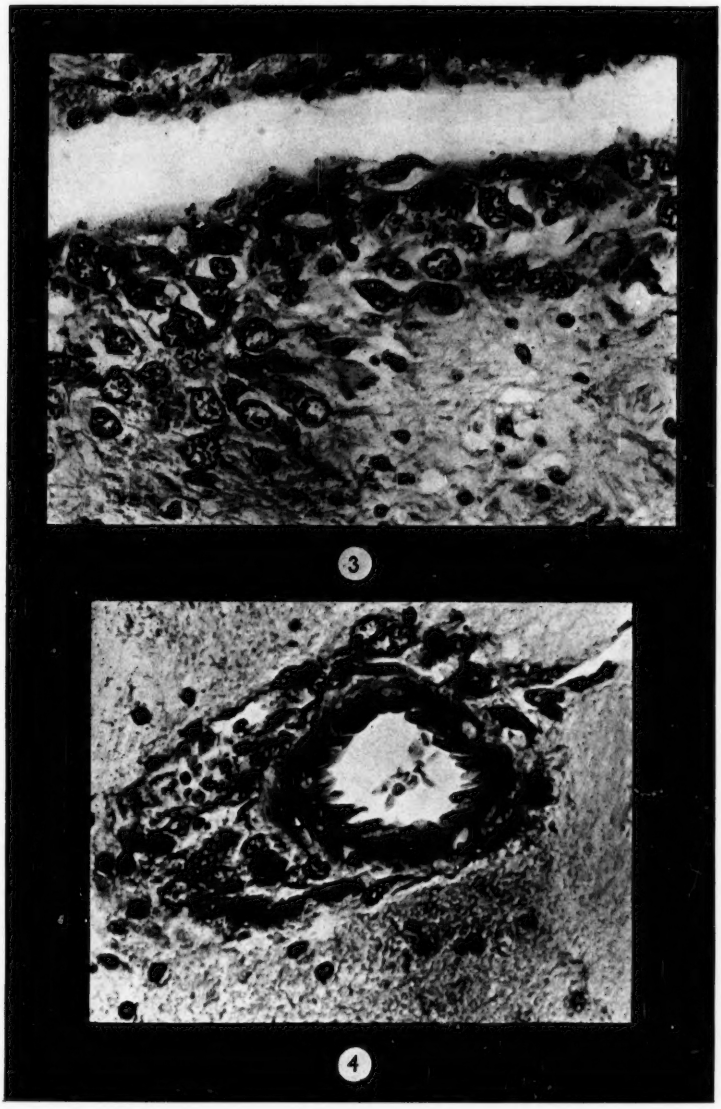

FIG. 3.—Ependymal and subependymal lesions at second serial intracerebral passage of myxoma virus.

FIG. 4.—Greatly enlarged cells in Virchow-Robin space of a cortical artery at second serial intracerebral passage of myxoma virus.

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Regional lymph-nodes.—Though frequently enlarged and congested, and sometimes showing petechiæ or small necrotic foci infiltrated with polymorphonuclears, none could have been diagnosed as myxomatous. In 2 cases syncytial masses of reticulum cells, forming a species of giant-cell, occurred at the periphery of the glands in far greater number than was ever seen in normal rabbits. At the hilum the adventitial cells of the vessels were often in active proliferation, without, however, assuming the appearance of myxoma-cells. Similar proliferation obtained in the capsule of some glands.

Spleen.—Here again no typically myxomatous lesions existed. The enlargement of the organ, most marked when sepsis was present, was partly due to great congestion and dilatation of the sinuses of the pulp. In some cases areas of the pulp showed increase of reticulum cells, but these were not greatly enlarged, and their nuclei were invariably pale rather than hyperchromatic. The Malpighian bodies were normal or, like the lymph-glands, contained small necrotic areas infiltrated with polymorphonuclears.

Liver.—In 3 of 16 cases the walls of the central veins were necrotic, and the surrounding liver-cells degenerating in the manner previously described (Hurst, 1937). In one of these, the animal with the necrotic adrenal described below, several branches of the portal vein were filled with organizing thrombus. In this case also the nuclei of some liver-cells were greatly enlarged.

Adrenal.—In one case inoculated intratesticularly, the left adrenal was macroscopically much enlarged (see above). On section the brownish central area represented a gland necrotic *in toto*, and the peripheral zone a broad band of young connective tissue, not characteristically myxomatous, with vascular changes like those of the skin. The necrosis of the whole gland must have been due to thrombosis, though the blocked blood-vessel was not seen in the preparations.

Testis.—In the one gland examined (14 days) after intratesticular inoculation, the parenchyma was in places wholly necrotic and the interstitial tissues the seat of much connective-tissue overgrowth, with deposition of abundant new collagen. Concentric thickening of the vessel-walls due entirely to endothelial proliferation was very conspicuous in some vessels, but none of the proliferated cells was enlarged to anything like the size common in unmodified myxoma.

Nervous system.—In 2 cases inoculated peripherally with neurovirus no lesions were detected.

Other organs.—No lesions were seen.

COMMENT AND SUMMARY.

On serial intracerebral passage, the myxoma virus underwent a well-defined modification which appeared to take place rather gradually. In the majority of instances the disease evoked by intradermal inoculation of the altered virus (neuromyxoma) was non-fatal; symptoms increased in severity up to the 9th–12th day, after which recovery set in. Most of the rabbits which died did so from secondary causes after the peak of the infection was passed. No animal inoculated by any route with even a minimal infective dose of the testicular virus used to initiate the series of brain passages survived more than

9 days ; the passaged testicular virus remains as virulent as ever. In circumstances more than usually favourable to multiplication, *e. g.* after intratesticular inoculation, the neurovirus usually killed. Possibly, with continued passage, the disease following intracerebral inoculation is proving fatal more often than immediately after the modification of virus had been established. Two testicular passages did not cause the virus to revert to its original characters.

Though milder in its manifestations than ordinary myxoma, with lesions somewhat different to the naked eye, the disease produced by the neurovirus was, clinically, undoubtedly myxomatous. Not so the microscopic picture. While epithelial changes were much the same as in classical myxoma, and all the alterations previously described in mesodermal structures (Hurst, 1937) were represented in a modified form, the striking transformation of various mesodermal elements into myxoma-cells, on which histological diagnosis usually depends, was wholly lacking in all situations. The new-formed cells in the dermis and meninges resembled more closely young fibroblasts, yet the lesions had not the appearance of those due to the Shope fibroma virus (OA strain of Andrewes, 1936 ; those caused by the IA strain have not yet been studied). In the brains at various passages it was possible to follow the gradual evolution of one type of cellular reaction from the other. The disease under consideration is, therefore, not only a milder myxoma ; it is also a definitely modified myxoma. It was interesting to find that, at the earlier passages, the subpial glia, ependyma and subependymal glia might develop in a direction morphologically rather similar to that taken by myxoma-cells. When stained with muci-carmine, none of the lesions afforded a positive reaction.

Lesions in the nervous system were mainly localized in the meninges, the vascular adventitial sheaths, the subpial glia, the ependyma and subependymal glia of the ventricular system, and the choroid plexuses. In a proportion of cases, meningitis was far more severe over the lower cord than elsewhere, and vascular changes here were responsible for partial softening of the cord with paralysis of the posterior part of the body ; this occurred in 7 of 47 cases, of which many were killed before the latest stage at which paralysis might occur. Except in the lower dorsal and lumbar spinal ganglia of such cases, where the neurons seemed to have been attacked directly by the virus, no definite lesions of nerve-cells other than those obviously due to vascular causes were detected. Moreover, after peripheral inoculation, no increased tendency on the part of the virus to invade the nervous system was discerned ; in no cases were nervous symptoms seen, and in 2 cases no lesions were discovered microscopically. Though not wholly devoid of affinity for the specific nervous structures, and especially for the ependyma and glia, the ordinary myxoma virus and the modified virus as studied after a score or so of brain passages are not outstandingly neurotropic according to the definition already given (Hurst, 1936).

Further consideration of the significance of this change can conveniently be deferred until various other observations have been published.

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MYXOMA AND THE SHOPE FIBROMA. III: MISCELLANEOUS OBSERVATIONS BEARING ON THE RELATIONSHIP BETWEEN MYXOMA, NEUROMYXOMA AND FIBROMA VIRUSES.

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THE complex of viruses dealt with in this series of papers at present includes four entities differing more or less in their pathogenic properties. They are (*a*) the classical myxoma virus, (*b*) the variant of this produced by serial cerebral passage (Hurst, 1936*c*), (*c*) the original "tumour-producing" strain of fibroma virus (OA strain of Andrewes, 1936), and (*d*) the mutant inflammatory strain of fibroma virus (IA strain of Andrewes). In his second and later articles on fibroma virus, Shope (1932, 1936*a*, 1936*b*) showed clearly that a relationship exists between (*a*) and (*c*), and the recent remarkable observations of Berry and Dedrick (1936), as well as those described herein and elsewhere, prove that all these viruses are, so to speak, variations on a single theme. Yet so dissimilar, clinically and/or pathologically, are the morbid conditions produced by different members of the group, that the relationship could hardly have been demonstrated apart from investigations in the immunological field. The following account details some points of similarity and of difference in the pathogenic activity of these viruses, which may assist in determining more exactly the relation each bears to the others.

DISTRIBUTION IN VARIOUS ORGANS.

(a) *Classical myxoma virus.*—Table I shows the concentration of virus in various viscera at different periods after intratesticular inoculation.

The virus used has already been described (Hurst, 1936*b*). To eliminate, as far as possible, virus-containing blood from the organs, the vascular system of each rabbit was washed out with 4 litres of saline; the technique has been described previously (Hurst, 1936*a*); it is far from effective for the spleen and bone-marrow, but is satisfactory for other viscera. The concentration of virus in the organ-suspensions was determined by intradermal inoculation of serial 10-fold dilutions in the backs of fresh rabbits; despite generalization at the usual time, the results were read quite easily. To avoid possible inhibition or obscuration of the reaction due to minimal quantities of virus, in the presence of lesions caused by large doses, the series of low dilutions from each rabbit was inoculated into one animal and that of higher dilutions into a second.

The results amplify those of previous workers and show how widespread throughout the body is the virus; as the disease progresses, the blood and thoracic and abdominal viscera all contain large quantities, and the nervous system appreciable amounts. At an early stage it was noteworthy that the lumbar cord always became infective before, or contained virus in higher

TABLE I.—*Distribution of Myxoma Virus in Organs of Rabbits following Intra-testicular Inoculation.*

Days after inoculation.	Strength of infecting suspension.	Titre of virus in— Blood.	Liver.	Lung.	Spleen.	Kidney.	Adrenal.	Mesenteric lymph-nodes.	Olfactory bulbs and frontal cortex.	Medulla.	Lumbar cord.
2	0·1 p.c.	10^{-1}	—	—	—	—	—	—	0	0	0
2	0·1 p.c.	10^{-1}	—	—	—	—	—	—	0	0	0
3	0·01 p.c.	10^{-1}	10^{-4}	10^{-4}	10^{-4}	10^{-3}	10^{-1}	10^{-2}	0	0	10^{-1}
3	0·1 p.c.	10^{-2}	—	—	—	—	—	—	0	10^{-1}	10^{-3}
3	0·1 p.c.	10^{-1}	—	—	—	—	—	—	0	0	10^{-3}
4	0·01 p.c.	10^{-2}	—	—	—	—	—	—	10^{-2}	10^{-2}	10^{-3}
4	0·01 p.c.	10^{-1}	—	—	—	—	—	—	0	0	10^{-1}
4	1·0 p.c.	10^{-2}	10^{-4}	10^{-5}	10^{-5}	10^{-4}	—	10^{-4}	$10^{-2}+$	—	$10^{-2}+$
5	1·0 p.c.	10^{-3}	10^{-4}	10^{-5}	10^{-5}	10^{-5}	10^{-4}	10^{-5}	$10^{-2}+$	—	$10^{-2}+$
7	0·01 p.c.	10^{-4}	10^{-6}	10^{-6}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-3}	10^{-2}	$10^{-4}+$

For the solid organs, dilutions are stated in terms of the wet weight of fresh material; thus 10^{-1} signifies a 10 p.c. suspension, and so on. Vascular system of all rabbits washed out with 4 litres saline.

+ following dilution indicates that end-point was not reached. 0 = No virus detected. — = No test made.

concentration than, higher regions of the nervous axis including the olfactory bulbs. There was thus no evidence that the myxoma virus reaches the central nervous system by the route taken by the equine encephalomyelitis virus (Hurst, 1936*a*). Rather, the findings suggest spread along the peripheral nerves, which in view of the apparently slight neurotropic affinities of the myxoma virus (Hurst, 1936*c*) is somewhat unexpected; opportunity has not yet arisen for further investigation of the matter by inoculation of virus into a previously denervated area.

(b) *Neuromyxoma virus.*—Though distributed similarly to the preceding, the modified virus apparently multiplies to a less extent (Table II). By the 9th day (after which the clinical condition may further deteriorate, owing no doubt to secondary influences) virus was localized in the lung and regional lymph-nodes. No evidence was forthcoming of increased affinity for the central nervous system of virus inoculated intratesticularly.

TABLE II.—*Distribution of Neuromyxoma Virus in Organs of Rabbits following Intratesticular Inoculation of a 1 p.c. Suspension.*

Days after inoculation.	Titre of virus in— Blood.	Liver.	Lung.	Spleen.	Kidney.	Adrenal.	Olfactory bulbs and frontal cortex.	Medulla.	Lumbar cord.
2	0	0	0	0	0	0	0	0	0
3	10^{-1}	—	—	—	—	—	0	0	0
4	10^{0}	10^{-1}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	0	0	0
6	10^{-1}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	0	0	0	10^{-3}
9	0	0	10^{-1}	0	0	0	0	0	0

In a further rabbit inoculated intradermally, virus was present in the regional lymphnodes, but not in the spleen, 11 days after inoculation. Vascular system of all rabbits washed out with 4 litres saline.

(c) *and* (d) *OA and IA strains of Shope fibroma virus.**—Probably no very material difference exists in the distribution of these two viruses (Tables III and IV). In both cases early generalization of virus occurs, and its titre in many organs is higher than in the blood. Neither strain of virus was detected in the nervous system. By the 7th day after intratesticular inoculation, virus is found only irregularly outside the primary lesion. In one case inoculated intradermally in about 30 areas with OA virus, the animal became greatly emaciated and the tumours failed to regress in the usual time; after 108 days abundant virus was still present in the primary lesions.

TABLE III.—*Distribution of OA Virus in Organs of Rabbits at an Early Stage of Infection following Intratesticular Inoculation of a* 10 *p.c. Suspension.*

Days after inoculation.	Presence of virus in— Blood.	Liver.	Lung.	Spleen.	Kidney.	Adrenal.	Regional lymph-nodes.	Frontal cortex.	Lumbar cord.
3	10^{0}	10^{-1}	10^{-2}	10^{-2}	0	10^{-2}	10^{-2}	0	0
4	10^{0}	10^{-2}	10^{-2}	10^{-3}	0	10^{-2}	10^{-2}	0	0
5	10^{-1}*	10^{-1}	10^{-2}	0	10^{-1}	10^{-1}	10^{-1}	—	—
7	0	0	10^{-1}	0	10^{-1}	0	10^{-2}	—	—
7	0	0	0	0	0	0	10^{-3}	0	0
7	0	0	0	0	0	0	0	0	0

* An asterisk in this and the following table indicates that while a reaction was obtained with the dilution stated, none occurred with more concentrated suspensions (see text).

TABLE IV.—*Distribution of IA Virus in Organs of Rabbits at an Early Stage of Infection following Intratesticular Inoculation of a* 10 *p.c. Suspension.*

Days after inoculation.	Titre of virus in— Blood.	Liver.	Lung.	Spleen.	Kidney.	Adrenal.	Regional lymph-nodes.	Frontal cortex.	Lumbar cord.
3	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-2}	0	10^{-1}	0	0
4	10^{-1}	0	10^{-2}	10^{-1}	10^{-1}	0	10^{-2}	0	0
5	0	0	10^{-2}	10^{-3}	0	10^{-2}	10^{-1}	—	—
7	10^{-2}*	0	10^{-1}	0	0	0	0	—	—
7	0	0	0	0	0	10^{-2}*	0	0	0
7	0	0	10^{-2}	0	10^{-2}*	0	10^{-1}	0	0

* For these I am indebted to Dr. C. H. Andrewes; they were passed intratesticularly several times in rapid succession before use in the above work.

Some interesting points were noted in connection with the skin-lesions in rabbits used for titrations. Suspensions of the regional lymph-nodes usually produced tumours a day or two sooner than those of other organs, but these never reached a large size. On the other hand, suspensions of lung gave tumours much larger than those of other organs containing the same number of M.I.D. virus. When at the 7th day titration of the organs yielded highly erratic results, a transient erythematous reaction had sometimes appeared on the 3rd or 4th day without going on to tumour-formation; similar behaviour has been observed in the case of neutral serum-virus mixtures. In a few instances at this stage the highest concentrations of blood or of an organ-suspension

gave negative results, when with further dilution a tumour appeared ; whether this phenomenon indicated a loose linkage between virus and antibody in the test material was not determined, though in a number of cases examined antibodies could be detected in the blood at this period.

Summary.—Like myxoma, fibroma is in the early stages a generalized infection ; it differs from myxoma chiefly in showing no apparent tendency to invade the central nervous system. The titre of virus in the blood and organs in fibroma and neuro-myxoma is never as high as that in the corresponding material in classical myxoma.

THE DEVELOPMENT OF PARTIAL IMMUNITY TO MYXOMA IN FIBROMA-INFECTED RABBITS.

Shope (1932) showed that the great majority of fibroma-immune rabbits infected with myxoma virus suffer a non-fatal infection, and again (1936*b*) that this partial resistance is apparent at least as early as the 14th day. For experiments such as those detailed in the next section of this paper, it seemed important that more precise information on the time of development of this immunity should be available.

Thirty to 70 days after infection with either the OA or the IA strain of fibroma virus, myxoma virus was inoculated intradermally into 12 rabbits ; the skin areas selected were distant from those previously infected. In 7 rabbits the new experiment took the form of a titration of virus, with an equal number of normal animals as controls ; in 3 the end-points were the same as in the controls, in 3 they occurred with the dilution tenfold lower, in 1 no reaction was noted even with the top dose of 1000 M.I.D. In 2 of the fibroma-immune animals and 2 of the controls the top dose of myxoma virus was only 10 M.I.D., so that the phenomenon of inhibition of small inocula in the presence of massive infections cannot have influenced the result ; in all 4 the end-point was the same. It can be said, therefore, that though some fibroma-immune rabbits fail to respond to a minimal dose of myxoma virus, resistance to larger doses is rarely encountered. These results agree with those of Shope. The macroscopic and microscopic appearances of myxoma in fibroma-infected or fibroma-immune rabbits will be related in a subsequent paper.

Experiments were then designed to determine how soon this refractory state to myxoma develops : Twelve rabbits were inoculated intradermally in five areas on one side of the back with 10 p.c. highly virulent (testicular) fibroma virus (OA strain). After various intervals 1 p.c. myxoma virus was inoculated into the other side of the back. The experiments are summarized in Table V, and show that, even when the interval was as short as 2 days, the reaction to myxoma was usually modified. There was, however, some individual variation in the rabbits ; thus 1 inoculated after an interval of 5 days developed typical myxoma and died within the short period of 6 days. On the other hand, 1 animal was almost wholly refractory on the 4th day and 1 completely on the 7th day to the large dose of myxoma virus injected—an event which, as we have seen, is unusual in rabbits completely recovered from fibroma. None of the animals killed for histology was seriously ill at the time

of sacrifice, and almost certainly all would have recovered; the remainder died or were killed when recovery was complete.

TABLE V.—*Results of Inoculating Myxoma Virus into Rabbits with Active Fibromata.*

Interval after inoculating fibroma virus.	Number of rabbits.	Result.
2 days	2	Killed for histology 15th and 16th days.
3 ,,	3	1 died 13th day; 2 recovered.
4 ,,	2	1 killed for histology 12th day; 1 transient reaction from 3rd to 7th day.
5 ,,	1	Died 6th day of typical myxoma.
7 ,,	2	1 killed for histology 26th day; 1 no reaction.
9 ,,	1	Killed for histology 24th day.
11 ,,	1	,, ,, 22nd ,,

Even when myxoma and fibroma viruses were inoculated simultaneously into the same or separate areas (Table VI), occasional animals survived or died at a very late stage from secondary causes. When separate simultaneous inoculations were made, the tumours appeared first, but regressed rather rapidly in about 9 days. The myxomatous primary and secondary lesions were more "tumour-like" than usual, and soon exceeded in size those due to the fibroma virus.

TABLE VI.—*Results of Inoculating* 1 *p.c. Myxoma and* 10 *p.c. Fibroma Virus Simultaneously.*

In one area or separately.	Number of rabbits.	Result.
One area	1	Died 9th day.
,,	3	2 died 9th day; 1 died 20th day.
Separate areas	1	Recovered.
,, ,,	3	All died 9th day.

If neuromyxoma virus is inoculated 4 days before fibroma no tumours develop, and even after 3 days only slight lesions result from the latter.

Summary.—A partial refractory state to myxoma virus develops very rapidly in rabbits infected with Shope fibroma virus; some difference obtains in the response of different animals. This early immunological response should probably be borne in mind in connection with experiments designed to demonstrate the Berry transformation of fibroma into myxoma virus. Rabbits infected with neuromyxoma virus become refractory to fibroma virus in about 4 days. These reactions are not necessarily wholly specific, since, as is well known, the response to many acute infections may be modified in the presence of another.

RESULTS OF THE BERRY TRANSFORMATION EXPERIMENT WITH VARIOUS COMBINATIONS OF FIBROMA AND MYXOMA VIRUS.

We know that myxoma virus is quickly inactivated by heat; the most recent figures for the thermal death-point (Hyde, 1936) are 10 minutes at 55° C. Berry and Dedrick (1936) have shown that inoculation of (domestic) rabbits with a mixture of Shope fibroma virus (but not other viruses) and heat-inactivated myxoma virus results in typical myxomatosis transferable in series. These fully controlled experiments were successful with material heated for 30 minutes at 60° and 75° C., but not at 90° C. In a personal communication Prof. Berry states that it is not necessary to inoculate the living and dead virus together in the same area.

This remarkable observation was confirmed at the first trial, and though no direct attempt was made to analyse the phenomenon itself, it seemed that it might be of use in defining the inter-relationship existing in the group of viruses under consideration.

In the following experiments inactivation was carried out at 60° C., and the time allowed lay between 40 and 50 minutes. In 7 controls inoculated subcutaneously on one or more occasions with as much as 10 to 20 c.c. of the heated virus no sign of myxoma appeared, and no immunity to a small dose of active virus was subsequently detected. Three of these were inoculated both intratesticularly and subcutaneously, and the testes removed 6 or 7 days later produced no disease in fresh animals. In 1 rabbit, inactivated myxoma virus mixed with vaccine virus was inoculated subcutaneously and intratesticularly; no sign of myxoma developed in this or in the passage rabbit receiving the testis excised on the 6th day.

In view of the facts established in the preceding section, in the experiments with heated myxoma and living fibroma virus, an attempt was made not to overwhelm a small amount of newly-formed living myxoma virus by the immunological response to a massive fibromatous infection. The results of repetition of Berry and Dedrick's original experiment, and of similar experiments substituting the IA for the OA strain of fibroma virus, are seen in Table VII. In 2 of 3 tests (single rabbits) with the OA strain, in 2 of 2 experiments (single rabbits) with the IA virus, and in 1 of 4 animals in 2 experiments with a mixture of OA and IA strains in equal proportions, myxoma resulted and ended fatally in 3 cases; the passage animals receiving the testicles of all 5 positives, excised before the appearance of signs of myxoma, died of typical myxoma in standard time.

It so happened that both primary animals recovering after having shown signs of myxoma, had been inoculated with the IA strain of fibroma, but this may, of course, have been pure coincidence. In one case (Experiment 4) the IA virus and heated myxoma virus yielded a positive result when a mixture of the same heated material with the OA strain failed to do so (Experiment 2).

The initial skin lesions in the primary animals were indistinguishable from ordinary fibromata, but, in the positives, skin generalization, eye signs, etc., appeared in from 9 to 13 days. Both fresh and glycerinated viruses were effective or ineffective in producing a positive result, and in one test, myxoma developed in only 1 of 2 rabbits receiving identical material. These

TABLE VII.—*Results of Inoculating (Intradermally + Intratesticularly) Mixtures of Heated Myxoma and Living Fibroma Virus.*

Experiment.	Heated 10 p.c. myxoma virus added to—	Previous treatment of viruses.	Time of appearance of signs of myxoma and result.	Result of passing testicle excised on 6th to 9th day.
1	1/5th volume 10 p.c. OA virus	M. 2 months in glycerin F. 2 weeks in glycerin	Myxoma 13th day; death 14th day	Died 7th day.
2	1/20th volume 10 p.c. OA virus	M. Fresh F. 1 week in glycerin	0	0
3	1/25th volume 10 p.c. OA virus	M. 3 weeks in glycerin F. Fresh	Myxoma 9th day; death 15th day	Died 9th day.
4	1/20th volume 10 p.c. IA virus	M. Fresh F. 1 week in glycerin	Myxoma 10th day; recovery	Died 7th day.
5	1/25th volume 10 p.c. IA virus	M. 3 weeks in glycerin F. Fresh	Myxoma about 10th day; recovery	Died 7th day.
6	1/10th volume 10 p.c. OA + IA viruses	M. 3 weeks in glycerin F. 3 weeks in glycerin	0 0	0
7	1/50th volume 1 p.c. OA + IA viruses	M. Fresh F. Fresh	Myxoma 10th day; death 13th day 0	Died 7th day. 0

0 = No sign of myxoma.

observations suggest that the transformation of fibroma into myxoma virus* takes place during the activity of the former in the living animal, and that, either from too ready immunological response to fibromatous infection or from other causes, individual rabbits may not favour the change. Again, from the observations related in the preceding section, it might be deduced that myxomatous transformation must be well advanced at a very early stage after inoculation of the mixture, since 3 of 5 positive cases ended fatally. It may be mentioned that the inoculation of mixtures giving positive results in normal rabbits into fibroma-immune animals was uniformly negative; in 8 rabbits infected from 79 to 186 days previously with OA or IA virus no sign of myxoma developed, and passages from these were likewise negative. This observation again links the phenomenon with the biological activity of the fibroma virus in the living animal.

When similar experiments were conducted with heated neuromyxoma virus as the complementary substance quite different results were obtained (Table VIII). Experiments 1, 2, 3 and 6 were performed at the same time and with the same fibroma virus as Experiments 3, 4, 6 and 7 in Table VII, 3 of which yielded positive results. Nevertheless, none of the rabbits in the present series developed myxoma, and passage of the excised testicles was also invariably negative. When living neuromyxoma virus and a mixture of IA and OA viruses were inoculated together, a non-fatal condition resulted in 3 animals.

* I am assuming that Berry and Dedrick's interpretation of the facts is correct, as certainly seems to be the case from the evidence available.

TABLE VIII.—*Results of Inoculating (Intradermally + Intratesticularly) Mixtures of Heated Neuromyxoma with Living Fibroma Virus.*

Experiment.	Heated 10 p.c. neuromyxoma virus added to—	Previous treatment of viruses.	Result.	Result of passing testicle excised on 6th–9th day.
1	1/25th volume 10 p.c. OA virus	M. 2 weeks in glycerin	0	0
		F. Fresh	0	
2	1/20th volume 10 p.c. IA virus	M. 2 weeks in glycerin	0	0
		F. 1 week in glycerin	0	
3	1/50th volume 10 p.c. OA + IA viruses	M. Fresh	0	0
		F. 3 weeks in glycerin		
4	1/10th volume 10 p.c. OA + IA viruses	M. Fresh	0	0
		F. Fresh	0	
5	1/10th volume 10 p.c. OA + IA viruses	M. Fresh	0	0
		F. 3 weeks in glycerin	0	
			0	
6	1/50th volume 10 p.c. OA + IA viruses	M. Fresh	0	0
		F. Fresh	0	
			0	

In 3 experiments (9 rabbits) in which heated myxoma virus was mixed with 1/10th volume of living neuromyxoma virus and inoculated intradermally, no difference was noted between the skin-lesions produced and those resulting from neuromyxoma alone. Only 1 rabbit died (on the 10th day), 6 recovered, and 2 were killed on the 6th and 11th days respectively; from each of the last pair the regional lymph-nodes inoculated into 6 fresh rabbits still produced the macroscopic lesions of neuromyxoma, and only 1 of these passage animals died.

The converse experiment, performed twice by adding heated neuromyxoma to 1/100th volume of living myxoma and inoculating the mixture into 5 rabbits, was also negative in that myxoma fatal in standard time resulted in all.

Summary.—Both OA and IA strains of Shope fibroma virus mixed with heat-inactivated myxoma virus may give rise to myxomatosis when inoculated into normal (but not into fibroma-immune) rabbits; passage animals die of quite typical myxoma. So far, neither heat-inactivated nor living neuromyxoma virus has proved effective in bringing about this transformation. Admixture with heated myxoma virus does not alter the pathogenic properties of living neuromyxoma virus. Conversely, heated neuromyxoma virus added to living myxoma virus has no influence on the resulting disease.

Discussion on the foregoing observations will be deferred to a later paper.

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FURTHER STUDIES ON ANTIGENIC DIFFERENCES IN MOUSE ERYTHROCYTES.

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In a previous paper (Gorer, 1936) it was shown that antigenic differences in mouse erythrocytes could be detected by means of sera from immunized rabbits. In order to obtain sufficient quantities of antigenically uniform material for immunization and the performance of absorption tests, it is necessary to use pooled cells from individuals drawn from strains that have been genetically purified by continuous brother-sister matings. Three such strains were used, described as the agoutis, albinos and blacks.

Anti-agouti sera absorbed once with the cells of blacks still agglutinated the cells of agoutis and albinos. Two absorptions with the cells of blacks or a single absorption with cells from the other two groups exhausted the sera completely. The antibody (or antibodies) remaining after one absorption with the cells of blacks was referred to as antibody I, the cells capable of removing the antibody easily, viz. those of agoutis and albinos, were said to possess the antigen I(3), while the blacks, whose cells appeared to have less affinity, were said to possess the antigen I(2).

Anti-albino sera contained an antibody specific for the albino cells. The cells of agoutis, however, appeared to have slight affinity for the antibody concerned since repeated absorptions lowered the titre of the serum. The cells of blacks appeared to have no affinity for the antibody. On the other hand, animals selected from a heterogeneous stock (the ZS stock) could exhaust the serum completely with two successive absorptions. The albinos were said to possess the antigen II(3), the agoutis II(1), the blacks were negative, whilst the ZS had the antigen II(2).

Landsteiner and Levene (1928) found that the tendency of anti-M and anti-N sera to be absorbed non-specifically (*i. e.* by cells negative in the agglutination test) depended on the serum used and not upon the individuality of the cells. Since we had found no cells that could not absorb antibody I, it was impossible to say whether the effect was similar to that described by Landsteiner and Levene. In the case of anti-albino sera it could be shown that the effect of absorption depended on the genetic constitution of the cells and could not therefore be described as non-specific—at least as regards a comparison of the agoutis and blacks. The ZS stock was very heterogeneous, and the effects obtained might have been due to the presence of antigen II(3) in a heterozygous state. Unfortunately the strain is now extinct, but the effects of hybridization between albinos and blacks are reported in this paper.

Further light has been thrown on the question of " non-specific " absorption by the investigation of anti-black sera.

EXPERIMENTAL RESULTS.

Technique.

Full technical details were given in the previous paper. For the preparation of anti-black sera the cells were washed and suspended in a strong anti-agouti serum (diluted 1/10) prior to injection, instead of in normal rabbit serum as was done previously. It is not certain that this alteration made any difference to the type of serum obtained. Some fresh anti-albino sera prepared by this method gave results very similar to those obtained when the cells were suspended in normal serum. The rabbits were bled after receiving 5 weekly injections.

For absorption two volumes of serum (usually diluted 1/20) were incubated with one volume of cells. Agglutination tests were performed by allowing equal volumes of cells and serum to sediment in small tubes. As a standard procedure 0·07 c.c. of each are used; under these conditions sedimentation is usually complete in one and a half to two hours; readings can be made after two hours; nothing is gained by prolonging the test beyond this period. All experiments reported in this communication were performed at 37° C. It is possible to obtain satisfactory results at room temperature with anti-black sera. Anti-albino sera must be tested at 37° C.; although antibody II is easily absorbed at lower temperatures, it gives better agglutination at 37° C.

Results Obtained with Anti-black Sera.

The results of over 20 experiments are shown in the tables in condensed form, as was done in the previous paper.

Four sera of this type have been prepared; all gave very similar results, but one had the disadvantage of being somewhat weak, whilst it was very difficult to absorb the species antibodies from another and only two were really suitable for extended investigation.

TABLE I.—*Anti-black Serum No. 3. Diluted 1/20 prior to Absorption.*

Whole experiment at 37° C.

Serum absorbed with cells of—	Number of times serum absorbed.	Tested on cells of—		
		Agoutis.	Albinos.	Blacks.
Agoutis	Once	—	—	a.c. or C.
,,	Twice	—	—	+++ to C.
Albinos	Once	—	—	+ to ++.
,,	Twice	—	—	—
Blacks	Once	—	—	—

a.c. and C. = almost complete and complete agglutination respectively.

Table I shows the results obtained with anti-black serum No. 3. It will be seen that the serum contains an antibody with a special affinity for the cells of blacks. A second absorption with the cells of agoutis lowers the titre slightly, but to a less extent than was the case with the antibody II of anti-albino serum. It will be noticed that after a single absorption with the cells of albinos, the agglutination given by the black cells is far weaker than when the absorption is done with agouti cells. A second absorption with the cells

of albinos exhausts the serum completely. This table also demonstrates that cells (here of albinos), though not agglutinable by a once absorbed serum, are yet able to absorb the agglutinin remaining in that serum.

This fact might suggest non-specific absorption, but since similar treatment with other (agouti) cells, does not remove the antibody, the suggestion of non-specificity may be discarded.

TABLE II.—*Anti-black Serum No. 4. Diluted 1/20 prior to Absorption.*

Whole experiment at 37° C.

Serum absorbed with cells of—	Number of times serum absorbed.	Tested on cells of—		
		Agoutis.	Albinos.	Blacks.
Agoutis	Once	— or tr.	tr. to. +	C.
,,	Twice	—	—	+++ to C.
Albinos	Once	tr.	tr.	+++ to a.c.
,,	Twice	—	—	tr.
Blacks	Once	— or tr.	— or tr.	— or tr.

N.B.—tr. = trace. For a *second* absorption with *agouti* cells it is sufficient to use 1 vol. of cells to 4 vols. of serum.

Table II shows the results obtained with serum No. 4. It will be seen that with this serum there is a slight difference between the cells of agoutis and albinos discernible after a single absorption with agouti cells. It appears that this serum, though of similar titre to No. 3, is rather more difficult to exhaust ; otherwise there is no essential difference between the two sera.

The cells of agoutis may be said to possess the antigen IV(1), those of albinos IV(2) and those of blacks IV(3).

It would appear from these experiments that the absorption of antibody IV in anti-black sera by albinos, and by inference that of antibody I in anti-agouti sera by blacks, depends upon the genetic constitution of the cells and cannot be said to be non-specific.

Experiments with Hybrid Stocks.

In these experiments albino males were crossed with black females and *vice versa*. There is no difference to be observed between the two types of cross ; members of this generation are referred to as F_1. F_1 animals were bred *inter se* to form the F_2 generation. F_1 animals crossed to albinos produced the A-backcross ; the B-backcross was derived by mating F_1 with black.

It was unfortunately impossible to make experiments with antibody I as all the anti-agouti sera had lost their potency. The present investigation is therefore confined to antigens II and IV.

Tests for antigen II were performed on about 30 F_1 animals and testing fluids* were prepared from five different anti-albino sera ; not all were used simultaneously, but frequently three were used in the same experiment. It was found that the reactions given by F_1 animals were, on the whole, distinctly

* Testing fluids are sera from which all antibodies but those characteristic of the pure line have been removed ; in this case anti-albino sera have been absorbed with the cells of agoutis or blacks.

weaker than those given by albinos. In the latter case, with a certain serum the reactions might be found to vary in intensity from " +++ to C " (complete agglutination), whilst with the F_1 the range of reaction would be from " + to +++ ". There is a certain amount of overlap between the two classes which may also be observed when the titre of a serum is measured. With weak sera a certain number of F_1 animals give negative or very feeble reactions; however, if absorption tests are performed, all F_1 animals can be shown to possess antigen II(3).

In agglutination tests for antigen II in the B-backcross animals at least three anti-albino testing fluids were used in every experiment, and in addition an absorption test with anti-albino serum was performed. In most cases it is possible to obtain about 0·2 c.c. of cells by bleeding a mouse from its tail ; with this quantity it is possible to obtain sufficient absorbed serum for tests on the cells of agoutis, albinos and blacks. The serum selected was one that had been found to give satisfactory results when absorptions were performed at room temperature and agglutination tests carried out at 37° C. In cases where only a small amount of cells was obtained, and thus only a very small volume of serum could be absorbed, it was found quite satisfactory to dilute the absorbed serum by half (the serum was diluted 1/25 prior to absorption). The volume of cells was judged by eye, and rather less than two volumes of serum was absorbed. In the earlier experiments controls were used from each of the three pure lines, but it was found that tests with agouti cells were redundant, and in later experiments only albinos and blacks were used. To make the control absorption tests rigorous a slight excess of serum was added to the albino cells, whilst in the case of the blacks there was an excess of cells.

Sixty B-backcross animals have been tested up to the present ; excellent agreement has been obtained between the different fluids, and in every case the agglutination and absorption tests have confirmed one another. In this generation 37 animals gave positive reactions and 45 were negative. The positive reactions were certainly no weaker than those given by F_1 animals.

Only 23 members of the F_2 generation have been tested so far. In this case, 17 animals gave positive results and 6 were negative. Fuller genetic details will be published later. In the B-backcross the agreement with a 1 : 1 ratio expected for a single dominant gene is within the range of experimental error. The figures obtained in the F_2 generation are as close as could be expected to a 3 : 1 ratio. It seems reasonable to assume that antigen II is determined by a single gene.

Tests for antigen IV have been made on the F_1, A-backcross and F_2 generations. The testing fluids were prepared from anti-black sera Nos. 3 and 4. All animals have been tested with both sera. The reactions given by the F_1 generation are slightly weaker than those obtained with the pure line of blacks. The difference is more clearly shown if the titres of sera are lowered by repeated absorptions with agouti cells. In one experiment (with serum No. 3) an excess of albino cells was accidentally used for absorption and the difference was specially noticeable. In most cases there was some overlap between the two classes and unless special methods such as successive absorptions or absorptions with an excess of cells are employed, the difference between the two classes is less striking than in the case of antigen II.

For tests with the A-backcross the sera must be absorbed twice with the cells of agoutis. An additional test with serum No. 3 absorbed once with albino cells was performed in many of the experiments.

The agreement between the different tests was excellent with one exception, which is not included in the figures given. The animal concerned was very stunted, and its cells appeared to be abnormal; it died shortly after being tested. Tests on 52 A-backcross mice have given 25 positive and 27 negative reactions. In the F_2 generation 24 animals have been tested, of which 19 were positive and 5 negative. Of the latter, 2 were negative for antigens II and IV. It seems justifiable to deduce that both antigens II and IV are determined by single genes. The presence of animals negative to both antibodies in F_2 shows that the genes are not allelomorphic.

In one series of experiments 23 F_2 animals and 13 A-backcross animals were tested with anti-black serum No. 3 absorbed *once* with agouti cells. In this case there was only one completely negative reaction (and that in F_2), although the albino controls gave a negative reaction (see also Table I). Individuals negative to twice absorbed serum gave reactions ranging from a faint trace (in the A-backcross) to " + " (in certain F_2 individuals); those positive to twice-absorbed serum gave complete or almost complete agglutination. This result does not invalidate the conclusion arrived at above; perhaps these sera contain a mixture of antibodies. It is not possible to make any statement about the genetic basis of these minor reactions.

DISCUSSION.

In a previous paper the question of the significance of quantitative differences was discussed. It was concluded that differences that were apparently quantitative might be due to slight chemical differences. Recent work by Landsteiner and Van der Scheer (1936) on the specificity of sera prepared against azoproteins shows a striking similarity to the results obtained with mouse erythrocytes. They found that repeated absorptions with azoproteins chemically similar to that used for immunization weakened the reactions given by the latter antigen. Antigens less closely related to the immunizing substance showed this effect slightly if at all.

On the other hand, the difference in the strength of reactions observed between homozygotes and heterozygotes reported in this paper is almost certainly quantitative. Scott-Moncrieff (1936) has demonstrated a somewhat similar quantitative effect in *Primula sinensis*. In this plant the gene " Dz " governs the production of an anthocyanin, pelargonidin 3-monoside; when the gene is heterozygous the amount of anthocyanin produced is much less than in homozygotes. The same paper contains many interesting examples of the complex way in which genes may interact to produce molecular modifications.

It will probably be some time before we are in a position to investigate the chemical basis of the serological differences found in mouse erythrocytes, but a study of Scott-Moncrieff's paper suggests many interesting parallels between the genetic basis of flower colour and that of the antigenic differences here described. It would appear that one gene determines chemical structure

in such a way that the cells are agglutinated by a given serum ; when this main gene is absent, minor reactions may be given if certain other genes are present. In order to study the genetic basis of such differences as those between antigens IV(2) and IV(1) it will be necessary to evolve a technique for determining the difference in single individuals ; experiments along these lines have given promising results.

SUMMARY.

1. By the employment of immune rabbit sera it has been possible to detect fresh antigenic differences between three pure lines of mice (agoutis, albinos and blacks). The blacks possess the antigen IV(3), the albinos IV(2), and the agoutis IV(1).

2. In some cases antibodies remaining in a once-absorbed serum can be removed by cells not agglutinated thereby. Pure lines tested with the same serum differ from one another in this respect, and therefore the phenomenon cannot be described as non-specific absorption.

3. Tests on hybrids between the albinos and blacks give results consistent with the hypothesis that positive agglutination reactions to antibodies II and IV are determined by two independent, incompletely dominant, autosomal (*i. e.* not sex-linked) genes.

4. The results reported up to date are considered in the light of Landsteiner and Van der Scheer's work on the immuno-chemistry of the azoproteins, and of Scott-Montcrieff's biochemical survey of genes governing the chemical structure of the anthocyanins.

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INFLUENZA VIRUS ON THE DEVELOPING EGG: IV.* THE PATHOGENICITY AND IMMUNIZING POWER OF EGG VIRUS FOR FERRETS AND MICE.

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In the first paper of this series (Burnet, 1936*a*) a brief account was given of the changes in pathogenicity of influenza virus in the course of egg passage. The present paper is concerned with a more detailed study of the results of inoculating mice and ferrets with fully-adapted egg virus of 65th to 74th passages, particularly in reference to the immunity developing after such inoculations.

METHODS.

Ferrets were inoculated intranasally under ether anæsthesia with about 0·25 c.c. of supernatant fluid from a typical egg membrane ground with 5 c.c. of broth and centrifuged. Temperatures were taken twice daily for 5 days after inoculation. Except on one occasion, noted below, the experimental ferrets were kept in a well-warmed room during the winter months. When serum was required, this was obtained by cutting off the extreme tip of the tail under light anæsthesia.

Mice were inoculated intranasally in the usual way under anæsthesia. Unless they were being kept for tests of immunity, survivors were killed and examined for lung lesions from 5 to 7 days after inoculation. All mice dying were examined; nearly all showed typical complete or almost complete consolidation of the lungs. The few non-specific deaths are excluded from the tables.

Antibody titrations in mice were carried out by mixing serial tenfold dilutions of serum with undiluted sand and paper pulp filtrate from two completely consolidated lungs ground with 10 c.c. of broth and subjected to a preliminary centrifugation. With very weak sera a mixture of undiluted serum with 1 : 10 virus was also used. The titre was expressed in terms of a standard serum, horse-serum IH2 from the National Institute for Medical Research, which was given an arbitrary titre of 2000. The procedure adopted was to evaluate the extent of the lung lesions with a figure from 0 to 4 (complete consolidation and death), and to obtain the titre by multiplying the serum dilution which showed incomplete protection by a factor—40 for very slight lesions, 20 for average grade 2 lesions, and 5 for nearly complete lesions

* The second and third papers of this series appeared in the *Australian Journal of Experimental Biology and Medical Science.*

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in surviving mice. This usually gave a value of approximately 2000 for the standard serum. If not, the values throughout the experiment in question were modified by the appropriate factor.

Antibody titrations on the chorio-allantois were carried out as described previously (Burnet, 1936*b*). The titre is expressed as the reciprocal of the serum concentration which, when added to an equal volume of virus, would reduce the pock count to 1 p.c. of the control, if it is assumed that both the percentage law and the C.P. constant relationship hold. Thus a serum which, diluted 1 : 100, gave 0·1 p.c. of " survivors " has a titre of 1000, and one which, undiluted, gave 10 p.c. of " survivors " a titre of 0·1.

THE PATHOGENICITY OF EGG VIRUS FOR MICE.

The egg-passage virus was originally derived from ferret material and has never been passed through mice. As has been previously pointed out (Burnet, 1936*a*), the egg strain has maintained its initial low grade of virulence for mice almost unchanged throughout. The following results were obtained when mice were inoculated with serial dilutions of a stock egg virus suspension (72nd passage) whose titre by egg titration was $4{\cdot}8 \times 10^5$ per c.c.

TABLE I.—*Lung Lesions in Mice Inoculated with Egg Virus.*

Egg-virus inoculum.	Lung lesions at 7 days.
Undiluted .	0, 0, 0*, 2, 2, 2
1 : 10 .	0, 0, 0, 0*, 0*, 3†
1 : 100 .	0, 0, 0, 0, 0, 1

* Lungs showing abnormal congestion, but no definite areas of consolidation.
† Heavy bacterial infection.

In all tables in which results of mouse inoculations are shown the following scale of lung lesions is adopted : 0, No areas of consolidation ; 1, small areas not exceeding in all 20 sq. mm. of lung surface ; 2, moderate areas up to half the total surface ; 3, half or more of the lung surface consolidated ; 4, complete or almost complete consolidation. Day of death of mouse shown in Tables II, III and IV by second figure.

The consolidated areas are not so deeply coloured nor so uniform in texture as similar areas produced by highly diluted mouse passage virus, but are distinct from anything observed with egg-membrane emulsions containing no virus. The congested but unconsolidated lungs noted in Table I are similar to lesions observed after inoculation with certain specimens of foreign serum, and, to a slighter extent, with emulsions of normal egg membrane.

By passage of the lung lesions produced by inoculating egg virus to mice, it is possible to obtain a virus highly active on mice and retaining much of its capacity to produce lesions on the egg membrane. This E.M. (egg-mouse) strain was developed in the fashion shown in Table II from 67th passage egg virus. The eggs of this passage contained dead embyros with typical brain hæmorrhages when opened on the third day after inoculation.

The virus was returned to the chorio-allantois after three mouse generations, and though it produced distinct lesions, these were poorly developed, and the embryos were alive and active when the eggs were opened at 3 days. The

TABLE II.—*Early Passages of E.M. Strain.*

Passage.	Lesions produced.
1. Egg (67th passage egg virus)	†3d. Hæmorrhagic encephalitis.
2. Mouse	0, 2, 3, 3
3. ,,	0, 0, 1, 2
4. ,,	3, 3, 4
5. Egg	Mild lesion.
6. Mouse	4^4, 5^4, 4^5, 4^5

supernatant fluid from one of these egg membranes contained a virus highly active against mice. Subsequently the virus has had the history shown over its 6th to 22nd passages :

$$E \rightarrow M \rightarrow 3E \rightarrow M \rightarrow 2E \rightarrow M \rightarrow 8E.$$

After the last egg passage shown the virus was still active in mice (4^4, 4^4, 4^6, 3+), but had failed to regain the initial pathogenicity for the egg. Embryos are found alive without lesions at 3 days, and the egg-membrane lesions are weak and unsuited for titration purposes.

IMMUNIZATION OF MICE WITH EGG VIRUS.

Egg virus is capable of inducing an effective active immunity in mice to which it has been administered intranasally. The following experiment indicates that surprisingly small amounts of virus may be effective :

Experiment 1.—The egg virus used was in the form of the usual supernatant fluid from one membrane ground up with 5 c.c. broth and centrifuged. Egg titrations showed that 0·05 c.c. of a 1 : 1000 dilution gave an average of 18 foci. Ten mice were inoculated intranasally under anæsthesia, with undiluted virus, and 6 each with 1 : 10, 1 : 100 and 1 : 1000 dilutions in normal horse-serum saline. Five days later 3 of the mice receiving undiluted virus were killed and their lungs examined. Two showed no lesions, the other very slight 1+ lesions. Twelve days after the immunizing inoculation all the mice were tested with active mouse virus in the form of a sand and paper pulp filtrate (1 mouse lung to 10 c.c. broth), half of each group receiving undiluted virus, half virus diluted 1 : 10. Six days later all mice not already dead were killed. Blood was obtained at the same time and pooled according to the original groups, Pool 1 from mice receiving undiluted egg virus, Pool 2 from those receiving 1 : 10, Pool 3 from the survivors of those receiving 1 : 100 or 1 : 1000 virus. The lung lesions observed in each mouse and the result of antibody titrations of the pooled sera are shown in Table III.

TABLE III.—*Immunization of Mice by Intranasal Administration of Egg Virus.*

Initial inoculation of egg virus.	Equivalent number of virus particles inoculated.	Result of inoculating mouse virus in dilution shown.			Antibody titre of pooled mouse serum.	
		Undiluted.	1 : 10.	1 : 100.	Mouse test.	Egg test.
0·05 c.c. undiluted	18,000	1, 2, 2, 4^4	0, 0, 0	..	500	6
,, 1 : 10	1800	0, 1, +	0, 0, +	..	3000	33
,, 1 : 100	180	0, 0, 4^6	1, 2, 4^6	..	2000	16
,, 1 : 1000	18	4^3, 4^5	0, 1, 4^4, 4^4	..		
Nil	0	4^4, 4^4, 4^5	4^5, 4^5, 4^6	4^-, 4^-, 4^5	..	..

\+ Non-specific deaths soon after test inoculation.

It will be seen that 14 out of 17 mice receiving egg virus diluted 1 : 100 or less were protected completely, or almost completely, while even with 1 : 1000

egg virus 2 mice were protected out of 6. It is of considerable interest that the mice receiving the largest amounts of egg virus were less effectively immunized than those receiving a 1 : 10 dilution. This is shown both by the results of the test inoculation and by the serum titrations, and is perhaps due to the associated presence of other antigens (chick embryo proteins) in the inoculum.

The immunity appears between the 7th and 10th days after inoculation.

Experiment 2.—Twenty-four mice received 0·05 c.c. of 1 : 10 stock egg-virus supernatant, and batches were tested with active mouse virus 3, 5, 7 and 10 days afterwards. The results are shown in Table IV.

TABLE IV.—*Development of Active Immunity after Intranasal Inoculation of Egg Virus Diluted* 1 : 10.

Time after inoculation.	Immunized mice.		Normal mice (controls).	
	Undiluted mouse virus.	1 : 10.	Mouse virus 1 : 10.	1 : 100.
3 days	4^2, 4^3, 4^4	4^3, 4^3, 4^4	4^3, 4^3, 4^5	4^4, 4^4, 4^4
5 ,,	*4*^{3}, *4*^{3}, +	4^3, 4^4, 4^6	4^3, 4^4, 4^5	4^4, 4^5, 4^5
7 ,,	*4*^{2}, *4*^{3}, +	4^3, *4*^{3}, 4^4	..	4^4, 4^4, 4^6
10 ,,	4^7, *4*^{3}, 0	0, 1, 2	4^4, 4^4	4^4, 4^4, 4^4

+ Non-specific deaths. *4*, Moribund or dead mice in which consolidation was not quite complete. See text.

Although mice inoculated with mouse virus 5 and 7 days after the egg virus inoculation failed to survive, several showed appearances in the lungs which probably indicate a slightly increased resistance. Control mice dying at the same time showed complete consolidation of the whole lung. In the immunized ones there was a narrow peripheral margin of unconsolidated lung, usually with a very sharp demarcation from the consolidated central regions.

In several other experiments, administration of 1 : 10 egg-virus intranasally has regularly induced resistance to about 100 lethal doses of active mouse virus. About half the mice show no lesions in the lungs after the test inoculation ; the others present small areas of consolidation.

IMMUNIZATION OF FERRETS.

It has been reported previously that the pathogenicity of egg virus for ferrets became progressively slighter with repeated egg passage. Ten ferrets have been inoculated with egg virus of 65th–67th passages in the present experiments. Three of these showed a significant rise of temperature above 103° F., but only one manifested symptoms ; the other 7 showed neither significant temperature changes nor symptoms. Subsequent tests with ferret-virulent virus (mouse strain) showed that all 10 animals were immune.

Experiment 3.—Three ferrets, F. 47, 48, 49, were inoculated intranasally with about 0·25 c.c. of stock egg virus. None showed symptoms, and their temperatures did not exceed 103° F. At 7 days after inoculation, a sample of blood was taken by cutting off the tip of the tail under anæsthesia, and the serum was subsequently titrated for influenza antibody content. Ferret 48 was accidentally killed at this stage. Another sample was taken at 14 days, and immediately afterwards both surviving ferrets and a normal control animal were inoculated intranasally

with virus of the mouse strain which had undergone three recent passages through ferrets. The normal ferret developed typical experimental influenza, the other two were unaffected. Antibody was present in small amount at 7 days, and in high titre at 14. Fig. 1 shows the temperature curves and the serological data.

The process responsible for the development of immunity following instillation of egg virus into the nose is not associated with the liberation of any considerable amount of virus in the nasal secretion. Washings from the noses of the 3 ferrets, F47, 48 and 49, were made on the 2nd, 3rd, 4th and 5th days after inoculation, by holding their noses beneath the surface of a small amount of broth for about a minute. Membrane filtrates from these washings were tested on the chorio-allantois with completely negative results throughout.

Even when ferrets infected with egg virus are killed in the early stages, very little virus can be liberated from the cells. Two ferrets, F52 and F53,

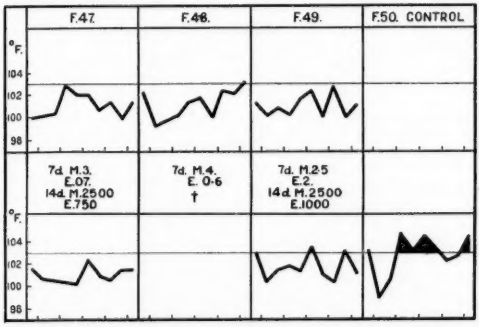

FIG. 1.—To show temperature response of three ferrets inoculated with egg-passage influenza virus (upper curves), and subsequent immunity 14 days later to inoculation with active virus (lower curves). Results of serum titrations in mice, M., and eggs, E., of samples of serum taken 7 and 14 days after the primary inoculation are included.

were inoculated intranasally with egg virus, and killed 3 and 5 days after inoculation. Emulsions were prepared from nasal mucosa and turbinates, lung and cervical lymph-glands by grinding the tissue finely with quartz powder and 4–5 c.c. of broth. These emulsions were centrifuged and the supernatant fluids tested on eggs for the presence of virus. Citrated plasma and washed blood-cells were also tested. Material from the nasal mucosa was the only one in which virus was detected. From the ferret (F53) killed at 3 days, eggs inoculated with undiluted supernatant fluid from the nasal mucosa gave 6/6, 5/5 and 3/5 specific foci. The other ferret, killed at 5 days, gave one typical focus only when similar material was tested on 3 eggs.

In another experiment, summarized in Fig. 2, one ferret was inoculated with egg virus and a normal uninoculated ferret placed in the same cage immediately afterwards. Two other ferrets were inoculated with egg virus which, by passage through mice, had developed a relatively high pathogenicity for this species (E.M. strain). One of the latter ferrets developed a significantly high temperature for a short period, but showed no symptoms and no secondary rise. The other animals showed only the usual random fluctuations. Serum

was obtained at the 14th day after inoculation from the contact animal and from the ferret F42, which showed no reaction to the E.M. strain. The contact ferret's serum contained no antibody, that of F42 a large amount. Tests for immunity were complicated by the fact that during the week in which they were carried out, all the ferrets had to be kept in an unheated room. The weather was unusually cold, and, as will be seen from the figure, the average temperature of the immune animals was at least 2 degrees below normal. For this reason, the upper level of normal temperature is taken as 101° F. instead of 103° F., as in the other charts. On this criterion the results are typical, the control ferret and the animal (F40) in contact with F39 showing significant fever and symptoms, the other 3 being immune.

An experiment to determine whether the mouse strain used in immunity tests was still infectious from ferret to ferret was made in connection with the experiment shown in Fig. 1. Twenty-four hours after the inoculations were

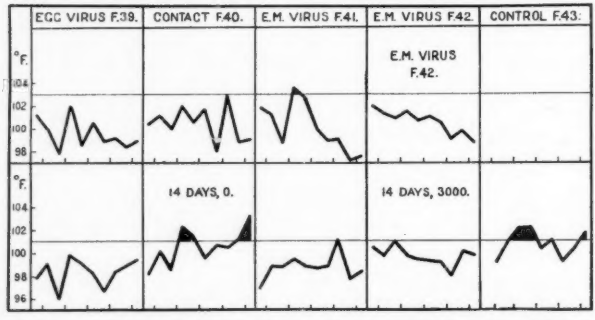

FIG. 2.—To show absence of infection by contact with a ferret which had received an immunizing dose of egg virus. The temperature curves show (upper) the response to primary inoculation or contact, and (lower) the response to a test inoculation of active virus. In the second set the significant temperature level is taken as 101° F. See text.

made another normal ferret, F51, was placed in the same cage as F50. Four days later it showed a maximum temperature of 104·5° F. and manifested the usual symptoms of nasal obstruction. Sera obtained 15 days after inoculation of F50 showed antibody titres of 15,000 for F 50 and 10,000 for F51 by mouse tests.

DISCUSSION.

The results of these experiments have been very consistent in showing that administration of egg-passage virus by the intranasal route gives rise to a solid immunity in ferrets and mice with very little clinical evidence of infection. In a little more than half the individuals of both species no evidence of infection other than the development of immunity was observed ; the others showed, in the case of ferrets, a small atypical temperature rise, or in mice, small areas of pulmonary consolidation.

As far as can be judged by limited experiments, very little virus is liberated into the nasal cavity during the egg-virus infection, and contact infection does not take place.

These results in the ferret are only of suggestive value in regard to the problem of protective inoculation in man, but they may serve to draw attention to the possibility of developing an infective, but innocuous, strain that would bestow a substantial immunity when administered by the natural respiratory route. Attempts along these lines may be associated with incidental accidents, and may possibly initiate epidemics, but seem more likely to provide a cheap and effective method of immunization than any method in which living or killed virus is administered parenterally.

SUMMARY.

1. Egg-passage influenza virus when administered intranasally to mice produces either no lesions or small areas of consolidation only, but effectively immunizes the mice against large doses of highly active mouse-passage virus.

2. Immunity can be produced by a very small dose of egg virus, and appears between the 7th and 10th day after inoculations.

3. Ferrets usually show no symptoms or temperature rise after intranasal inoculation of egg-influenza virus; occasionally an atypical single spike temperature rise occurs.

4. A high grade of immunity demonstrable either by direct inoculation or by titration of serum for specific antibody regularly follows such inoculation.

5. Unlike typical ferret influenza, the infection induced by egg virus is not infectious by contact to another ferret.

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INFLUENZA: FURTHER EXPERIMENTS ON THE ACTIVE IMMUNIZATION OF MICE.

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RECENT work has brought nearer the possibility of artificially increasing the immunity of human beings to influenza. It has been shown that mice can be immunized by inoculating them subcutaneously or intraperitoneally with living influenza virus (Smith, Andrewes and Laidlaw, 1935; Francis and Magill, 1935; Shope, 1936). But crude tissue suspensions containing living virus do not afford the best material for human vaccination, and experiments have accordingly been carried out with a view to obtaining the most favourable antigen for trial as a prophylactic in man. Objects in view

were, first, to obtain a suspension with as high a virus-content as possible, second, to purify this by freeing it from undesirable constituents, and finally, to inactivate the virus if possible without destroying its antigenic value.

ATTEMPTS TO INCREASE VIRUS-CONTENT OF FILTRATES.

The preparation and filtration of suspensions from infected mouse lungs have been described in previous papers of this series (Smith, Andrewes and Laidlaw, 1935). Unfiltered suspensions (W.S. strain) so prepared were found to contain 10^7 or 10^8 minimal infective doses per c.c.; filtrates of these contained only 10^5 or 10^6 m.i.d. per c.c. In hope of increasing the proportion of virus which would pass the filters, the crude tissue-suspensions were digested with trypsin before centrifuging and filtering. In other experiments they were extracted with 9 p.c. saline or with distilled water instead of with a mixture of broth and normal saline as usual. No increase of titre was obtained by these procedures, nor by means of minor variations in the technique of filtration such as varying the pH of the suspending fluid within the range allowed by the stability of the virus.

TECHNIQUE OF PREPARING FILTRATES.

Though these attempts to increase the virus titre were abortive, we evolved a standard procedure for preparing filtrates which will be briefly described as it yielded consistently potent preparations. Six mice were inoculated intranasally with 0·05 c.c. of virus filtrate under ether anæsthesia. They were killed with chloroform 2 days later and their lungs removed and weighed; usually about 1 g. of tissue was obtained. Lungs of mice killed as early as this might or might not show definite lesions, but their virus content was always high. The lungs were ground with powdered Pyrex glass and a mixture of equal parts of broth and saline added slowly to give a 5 p.c. suspension, which was centrifuged at low speed for 10 minutes and then filtered through a freshly-prepared asbestos bed. To prepare this bed, about 10 c.c. of wet asbestos pulp, made by shredding Seitz discs, was shaken up with 100 c.c. of water, and packed down on a sintered glass disc 5 cm. in diameter under a negative pressure of 20 cm. Hg. About 10 c.c. of broth were passed through the bed before use. Turbid lung suspensions were usually completely cleared by filtration through this, and could subsequently be filtered quickly through a gradocol membrane of average pore diameter 0·6 to 0·75 μ (Elford, 1931). Filtrates so obtained could if necessary be stored at $-2°$ C. for 1 or 2 weeks to furnish a supply of virus for subsequent passages; they were, however, always freshly prepared for experiments on immunization.

SPONTANEOUS INCREASE IN VIRUS TITRE.

Though we failed to increase our virus titre by modifications in technique, a fortunate chance produced for us the effect we desired. Filtrates of the W.S. strain of virus were titrated on many occasions between June, 1935, and December, 1936. Up to January, 1936, the average titre was 10^5 m.i.d. per c.c., but from April, 1936, onwards the titre was usually 10^7, less often 10^6; this

represents an increase of 10- to 100-fold. The increased titre was not due to any change in technique nor in the susceptibility of the mice. This seems fairly certain, for the swine influenza virus carried on by mouse passage at the same time retained its original titre (10^4 to 10^5 m.i.d. per c.c.) unaltered during this period ; it is true that it was passaged through mice less frequently than the W.S. strain. All the mice used came from one strain, bred at Mill Hill. It does not appear that the change in the W.S. virus was a gradual one occurring uniformly in the course of many mouse passages. Once it was adapted to infect mice in 1934, its behaviour remained much the same during 65 passages carried out during 18 months, and after the increase in titre between February and April, 1936, no further increase occurred in the course of 20 further passages between April and November, 1936. It seems rather that a change took place fairly suddenly between January and April, 1936. We suggest the possibility that a "mutant" virus appeared about January, 1936, and in the course of 6 or 8 passages during the next 3 months gradually supplanted the original form. Higher virus titres might of course be due to one of two causes : the virus might multiply more rapidly so that the mouse lungs and therefore filtrates actually contained more virus particles ; alternatively there might be no more virus particles present but these might be more aggressive, so that a smaller number would be required to produce infection. Though we cannot exclude the latter, we incline to the former alternative, particularly as later virus filtrates were more effective antigens than earlier ones, even when inactivated. We find in fact that mice can now be immunized more readily than was reported in earlier papers by ourselves (Smith, Andrewes and Laidlaw, 1935) and Francis and Magill (1935). The spontaneous change in virus behaviour is probably the explanation, and may ultimately prove most valuable in facilitating the immunization of human beings.

ATTEMPTS TO PURIFY VIRUS BY ADSORPTION AND ELUTION.

It seemed desirable to try to prepare a vaccine to be used on man as free as possible from undesirable constituents such as mouse-proteins. The method of adsorption and elution was first tried, as this has been employed with some success for purifying other viruses. Preliminary experiments showed that influenza virus was fairly stable between a pH range of 7 to 9, but that it was unsafe to keep it for very long on the acid side of pH 7. The virus was poorly adsorbed by kaolin either at pH 6 or at 7·2. Better results were obtained with suspensions of alumina (Willstätter, C.). When 3 c.c. of alumina suspension were shaken with 5 c.c. of mouse lung filtrate at pH 6 and centrifuged, the virus titre in the supernatant fluid was found to be less than 0·01 p.c. of the control held at the same pH. Smaller amounts of alumina and more alkaline conditions gave less satisfactory results. It was then sought to elute the virus with phosphate buffer having a pH of 8, and an active eluate was readily obtained having a protein content much below that of the original fluid. Unfortunately the fall in virus-content was of the same order as the reduction in the protein. In one experiment a filtrate having a titre of 10^6 m.i.d. per c.c. had its virus content reduced by adsorption

to 10^2 m.i.d. per c.c. ; the eluate obtained had a value of only 10^4. Successive elutions with buffer were attempted, following the favourable reports of Kligler (1934) with vaccinia virus, but the results were not encouraging.

PURIFICATION BY WASHING ON A MEMBRANE IMPERMEABLE TO VIRUS.

Elford with various collaborators has shown that partial purification of a number of viruses and bacteriophages can be effected by washing on a gradocol membrane which retains the virus while allowing unwanted proteins to pass. Influenza virus is always retained by a membrane having an average pore diameter of 0·15μ (Elford, Andrewes and Tang, 1936) ; accordingly a membrane with an A.P.D. of 0·10 or 0·12μ is suitable for washing this virus. A membrane holder with an open top was used and filtration carried out under negative pressure. The filter and the method have been described by Galloway and Elford (1936). The desired volume of virus-filtrate was placed in the filter ; the progress of filtration could be observed and fresh washing fluid added whenever approximately nine-tenths of the volume had passed through. In most experiments with influenza four washings were carried out ; 8 c.c. of virus filtrate could be thus washed in 3 or 4 hours.

In early experiments we washed with saline or with buffered phosphate ($M/30$ Na_2HPO–pH 7·6), but a stringy precipitate, possibly of nucleoprotein, was liable to form in the washed virus suspension. This could be avoided by using phosphate buffer at pH 8 as the washing fluid. Washed preparations were thus obtained free or almost free from protein as judged by the sulphosalicylic acid test and having a titre about one-tenth that of the original filtrate. They were, however, less stable than unwashed filtrates. A specimen of the latter when kept in a test-tube at −2° C. retained some activity for more than 2 months. Its original titre was 10^6 m.i.d. per c.c. ; this fell to 10^5 in 4 days, but had not fallen to 10^4 until after 36 days. On the other hand, a filtrate washed in phosphate and having an initial titre of 10^5 m.i.d. per c.c. had lost all activity after 13 days in the cold.

The addition of ½ p.c. Witte peptone to the washing fluid was found to increase the viability of the virus, but a precipitate slowly formed in the peptone-phosphate mixtures. We ultimately found that ½ p.c. Witte peptone in 0·9 p.c. saline (pH adjusted to 8) was a thoroughly satisfactory washing fluid. Virus washed with this proved as stable as when untreated, and on a number of occasions filtrates retained their full titre after four washings.

INACTIVATION OF VIRUS WITH FORMALDEHYDE.

Influenza virus inactivated by means of formaldehyde had already been found to be capable of immunizing ferrets (Smith, Andrewes and Laidlaw, 1935). It was found that filtrates of infected mouse lung were almost always inactivated by formaldehyde 1 : 10,000 in 5 days at −2° C. ; 1 : 5000 formaldehyde invariably produced inactivation in this time. No significant difference was found between the concentrations required to kill virus in centrifuged suspensions, filtrates or washed preparations. In order to test whether virus in formolized vaccine was completely inactivated, we inoculated mice intranasally with the undiluted vaccine and killed them after 3 to 5 days, to

examine their lungs for the characteristic lesions. In some instances we made passages from the normal-looking lungs of such mice, but we never recovered virus by this means. It is evident that influenza virus is more susceptible to inactivation by weak formaldehyde than are most viruses.

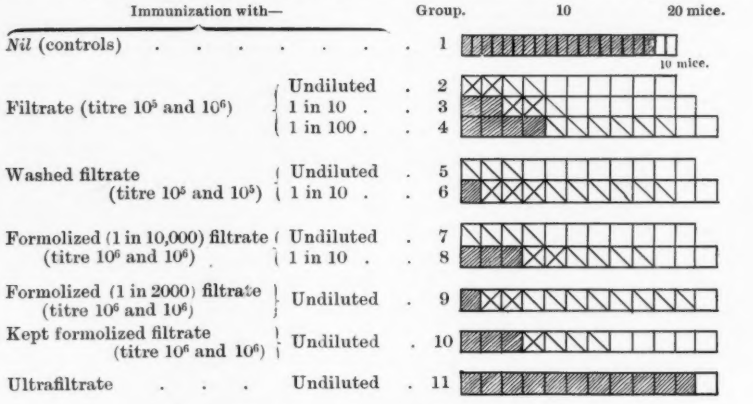
THE IMMUNITY EXPERIMENTS.

Plan.—Groups, usually of 12 mice of 12 to 14 g., were inoculated with the various preparations to be tested. Mice become less susceptible to infection as they grow older. Accordingly we always set aside one, or if necessary several, groups of control mice at the time the experiment was begun. All the mice in one experiment were thus of similar age at the time of the immunity test. In most experiments, following Francis and Magill's (1935) plan, mice received 0·2 c.c. subcutaneously, a further 0·5 c.c. intraperitoneally 14 days later, and were tested for immunity 14 days after this second dose. In the immunity test each mouse received 0·05 c.c. of virus filtrate intranasally under ether anæsthesia ; they were observed for 14 days and symptoms and deaths recorded, all dead mice being examined *post mortem* ; after 14 days the survivors were killed to determine the extent of pulmonary damage, if any. This method of immunizing and testing was followed in all the experiments recorded in the tables below unless otherwise stated. In most experiments the virus filtrates used for the immunizing doses and for the immunity test were titrated by testing each dilution on 3 mice. The relative antigenic value of different preparations was compared by immunizing groups with various dilutions of antigens. Charts I to VI show the results of 6 immunity experiments. Where in these charts it is stated that the titre of a virus was 10^5 and 10^6, the meaning is that virus used for the first immunizing dose had 10^5 and that used for the second immunizing dose 10^6 m.i.d. per c.c. Titres for formolized virus refer, of course, to the virus-titre before formalin was added.

The first two experiments (Charts I and II) were carried out in order to determine the effect of washing and of formolizing on the antigenic value of virus filtrates.

Experiment 1 (*Chart I*) shows that immunizing capacity fell as the dose was diminished, but that even 1 in 100 dilutions of filtrate produced some effect (groups 2 to 4). Washed virus was about as effective an antigen as unpurified filtrate (groups 5 and 6). Filtrate inactivated by 1 : 10,000 formaldehyde was apparently as effective as the living virus, but it must be noted that the virus-content of the first dose was higher than that of the first dose of live virus, and therefore more antigen was probably given (groups 7 and 8). From group 9 it appears that when the strength of formaldehyde was increased to 1 : 2000, the antigenic value was not destroyed. The mice in group 10 received at any rate some protection from formolized filtrate (1 : 10,000 formaldehyde) which had been stored in the cold (—2° C.) for 2 months. Finally in group 11 we tested the hypothesis that the immunizing agent in filtrates was not the virus itself but a soluble antigen ; the mice in this group accordingly received filtrate which had been freed from virus by passing through a membrane of average pore diameter 0·12μ. No protection was afforded by this ultra-filtrate.

CHART I.—*Exp. 1: Effect of Washing and of Formolizing on Antigenic Value.*


Immunization with—
Group.
10
20 mice.
Nil (controls) 1
10 mice.
Filtrate (titre 10^5 and 10^6) Undiluted . 2
1 in 10 . . 3
1 in 100 . . 4
Washed filtrate (titre 10^5 and 10^5) Undiluted . 5
1 in 10 . . 6
Formolized (1 in 10,000) filtrate (titre 10^6 and 10^6) Undiluted . 7
1 in 10 . . 8
Formolized (1 in 2000) filtrate (titre 10^6 and 10^6) Undiluted . 9
Kept formolized filtrate (titre 10^6 and 10^6) Undiluted . 10
Ultrafiltrate . . . Undiluted . 11

Titre of filtrate used for immunity test: 10^6.

Each horizontal column represents the outcome of the immunity test on a group of mice immunized as indicated.

▓ = mouse died of influenza within 14 days.

⊠ = mouse with extensive lung lesions when killed after 14 days.

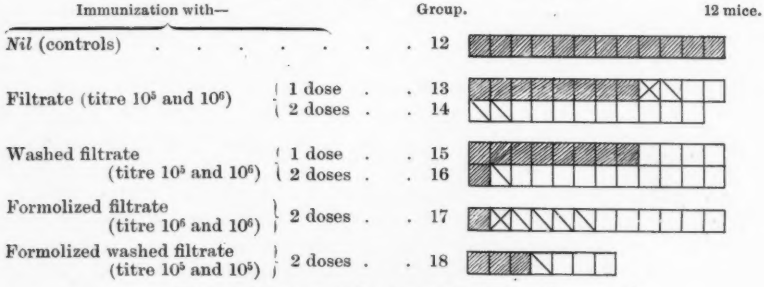
▨ = „ small „ „ „

□ = „ no „ „ „

For further details of experiments see text.

Experiment 2 (*Chart II*).—From this it seems that even one dose of filtrate or of washed filtrate can confer some immunity, but that two doses produce a far better result. The conclusion of Experiment 1 is confirmed that two doses of washed or of formolized virus are almost, if not quite, as effective as two doses

CHART II.—*Exp. 2: Effect of Number of Doses.*


Immunization with—
Group.
12 mice.
Nil (controls) 12
Filtrate (titre 10^5 and 10^6) 1 dose . . 13
2 doses . . 14
Washed filtrate (titre 10^5 and 10^6) 1 dose . . 15
2 doses . . 16
Formolized filtrate (titre 10^6 and 10^6) 2 doses . . 17
Formolized washed filtrate (titre 10^5 and 10^5) 2 doses . . 18

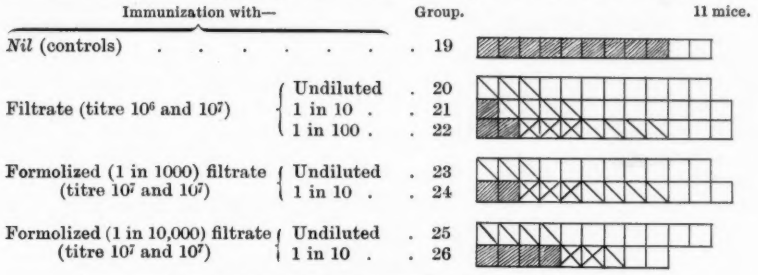
Titre of filtrate used for immunity test: 10^6.

See legend at foot of Chart I.

of untreated virus. From a small group (group 18) of mice it seems that virus which has been first washed and then formolized is less satisfactory; this matter will be considered later (*cf.* Experiment 4).

In Experiment 3 (*Chart III*) are compared the relative values of living filtrate, filtrate inactivated by 1 : 1000 formaldehyde and that inactivated by 1 : 10,000 formaldehyde. We wished to determine whether there was a

CHART III.—*Exp. 3 : Effect of Two Strengths of Formol on Antigenic Value.*

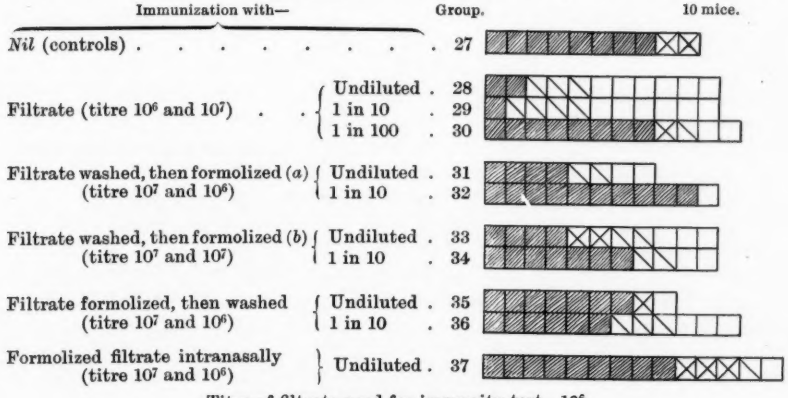

Immunization with—
Group.
11 mice.
Nil (controls) 19
Filtrate (titre 10⁶ and 10⁷)
Undiluted . 20
1 in 10 . . 21
1 in 100 . . 22
Formolized (1 in 1000) filtrate (titre 10⁷ and 10⁷)
Undiluted . 23
1 in 10 . . 24
Formolized (1 in 10,000) filtrate (titre 10⁷ and 10⁷)
Undiluted . 25
1 in 10 . . 26

Filtrate used for immunity test not titrated. Titre probably 10^6 or 10^7.
See legend at foot of Chart I.

wide zone of formaldehyde concentration in which inactivation of virus without destruction of antigenic power occurred, or whether we could only work in a very restricted range. It appears that 1 : 1000 formaldehyde, 10 times the strength necessary for inactivating, can safely be used for preparing a vaccine.

The results of Experiment 4 (*Chart IV*) were a disappointment to us. Washed virus had seemed almost as good an antigen as when untreated;

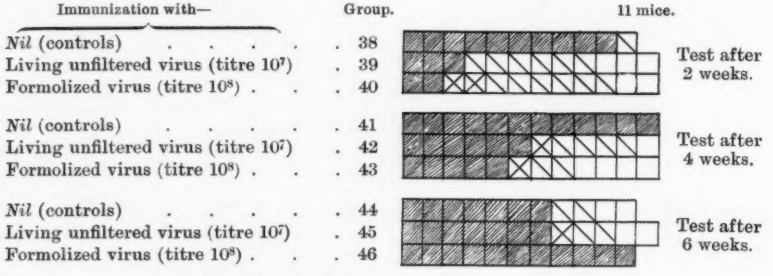
CHART IV.—*Exp. 4 : Effect of Combining Washing and Formolization.*


Immunization with—
Group.
10 mice.
Nil (controls) 27
Filtrate (titre 10⁶ and 10⁷)
Undiluted . 28
1 in 10 . 29
1 in 100 . 30
Filtrate washed, then formolized (a) (titre 10⁷ and 10⁶)
Undiluted . 31
1 in 10 . 32
Filtrate washed, then formolized (b) (titre 10⁷ and 10⁷)
Undiluted . 33
1 in 10 . 34
Filtrate formolized, then washed (titre 10⁷ and 10⁶)
Undiluted . 35
1 in 10 . 36
Formolized filtrate intranasally (titre 10⁷ and 10⁶)
Undiluted . 37

Titre of filtrate used for immunity test: 10^6.
See legend at foot of Chart I.

formolized vaccines reacted almost as well as living ones. Yet virus which was first washed and then treated with formaldehyde (1 : 5000) was significantly inferior. In the groups of mice 31 and 32 we used a single batch of vaccine for the two injections ; for those in groups 33 and 34 we prepared a fresh batch for the second dose in order to guard against the contingency that the formolized, washed antigen was unduly labile. As Chart IV shows, both antigens were poor, and when we formolized a filtrate first and then washed it, there was little if any antigenic power in the product (groups 35 and 36). The low antigenic value of washed and formolized virus is confirmed by the result in the mice of group 18, Chart II, and by yet another experiment of which the result is not included in the charts. The significance of these results will be discussed later. In this experiment is included also a group of mice (No. 37) which we attempted to immunize by means of two intranasal inoculations under ether anæsthesia of 0·05 c.c. formolized virus ; no immunity was produced.

CHART V.—*Exp. 5 : Duration of Immunity after One Dose of Unfiltered Virus.*


Immunization with—
Group.
11 mice.
Nil (controls) 38
Living unfiltered virus (titre 10⁷) 39
Formolized virus (titre 10⁸) . . . 40
Test after 2 weeks.
Nil (controls) 41
Living unfiltered virus (titre 10⁷) 42
Formolized virus (titre 10⁸) . . . 43
Test after 4 weeks.
Nil (controls) 44
Living unfiltered virus (titre 10⁷) 45
Formolized virus (titre 10⁸) . . . 46
Test after 6 weeks.

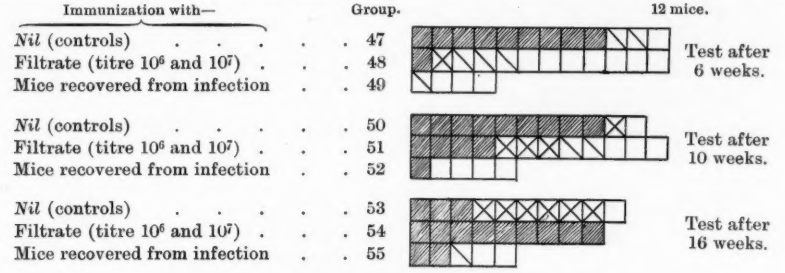
Titre of filtrate used for immunity test: 10^6 in each test.
See legend at foot of Chart I.

Experiments 5 *and* 6 (*Charts V and VI*) concern the duration of immunity. In Experiment 5 we used unfiltered 5 p.c. virus suspensions with and without treatment by formaldehyde (1 : 10,000), and studied the effect of a single subcutaneous dose of 0·2 c.c. Both living and formolized vaccines produced a definite immunity evident in the test made after 2 weeks ; groups 42 and 43, tested after 4 weeks, seemed to have a lower degree of immunity, and after 6 weeks the immunized groups (45 and 46) were not protected at all. In another uncharted experiment similar to this one, there was some evidence of immunity 6 weeks after a single dose of living virus (titre 10^8) or formolized virus (titre 10^8). In this experiment, 24/39 controls died after their immunity test, but only 2/17 of those receiving live virus and 2/10 of those immunized with formol vaccine.

Experiment 6 (*Chart VI*) shows that in mice receiving two doses of filtrate there was good immunity after 6 weeks, less after 10 weeks and none after 16 weeks. We tried in this experiment to compare the duration of immunity in vaccinated mice with that in mice recovered from an actual virus infection.

With this object, 48 mice in 4 boxes of 12 were inoculated intranasally with a 1 in 10,000 dilution of virus filtrate. One lot of 12 was killed off after 5 days in order to be sure that the strength of filtrate used would infect regularly ; all 12 of these mice showed extensive lung lesions. Unfortunately, only 14 of the remaining 36 mice recovered. These 14 were tested in lots of 4, 5 and 5 after 6, 10 and 16 weeks respectively (groups 49, 52 and 55). It appears that they were mostly immune after 6 and 10 weeks, but that there was doubtfully any immunity after 16 weeks. Of those surviving the immunity test, most showed old lung lesions, doubtless dating back to their first infection ; whole lobes or large parts of them were fibrotic and airless, with, in some instances, striking bronchiectasis. Such old lesions have not been recorded in the chart. The experiment was inconclusive, but suggests that after actual infection the immunity is not much more durable than after vaccination.

CHART VI.—*Exp. 6 : Duration of Immunity after Two Doses of Filtrate.*


Immunization with—
Group.
12 mice.
Nil (controls) 47
Filtrate (titre 10^6 and 10^7) . . . 48
Mice recovered from infection . . 49
Test after 6 weeks.
Nil (controls) 50
Filtrate (titre 10^6 and 10^7) . . . 51
Mice recovered from infection . . 52
Test after 10 weeks.
Nil (controls) 53
Filtrate (titre 10^6 and 10^7) . . . 54
Mice recovered from infection . . 55
Test after 16 weeks.

Titres of filtrates used for immunity tests: 10^7 (6 weeks).
10^6 (10 weeks).
10^7 (16 weeks).

See legend at foot of Chart I.

DISCUSSION OF RESULTS OF IMMUNITY TESTS.

We conclude from the results of all these experiments that subcutaneous or intraperitoneal inoculation of mice with living filtered or unfiltered mouse virus confers definite immunity against intranasal infection with many infective doses of virus. A single vaccinating dose confers some protection, but two doses given a fortnight apart are far more effective. Living virus which has been largely freed from impurities by washing seems to be as good an antigen as unpurified virus, though not demonstrably superior to it.

Virus, filtered or unfiltered, which has been inactivated by 1 in 5000 or 1 in 10,000 formaldehyde has an antigenic value as good as, or but little inferior to, that of living virus. From a glance at Charts I, II, III and V it would appear to be quite as good as living virus, but it chanced in all these experiments that the virus used to prepare the formolized vaccine had a titre rather higher than that of the comparable live vaccine. A number of facts suggest that when living virus is inoculated subcutaneously or intraperitoneally into mice, it does not multiply. First, the immunity induced is

proportional to the amount of virus injected; second, we have failed to recover virus from the lungs or noses of vaccinated mice, the two sites in which alone virus occurs in infected animals; and third, there is very little difference between the immunizing value of living and inactivated virus.

It is difficult to explain why virus which has been both washed and purified makes a relatively poor antigen. A possible analogy is afforded by the fact that it is difficult to make a good formol-toxoid out of purified diphtheria toxin, but in that instance trouble is avoided by formolizing first and purifying afterwards (Schmidt and Fjord-Nielsen, 1934). Yet one of the worst of all our antigens was one in which we formolized the virus first and washed afterwards. Moreover our washing fluid contained peptone, a substance which appears to stabilize a toxin. The matter remains obscure.

In the experiments here described we have used vaccines made of mouse tissues for immunizing mice. Shope has reported (1936) that mouse vaccines were relatively ineffective in immunizing ferrets and *vice versa*, unless the intraperitoneal route of inoculation was used. This might appear to decrease the hope of immunizing human beings with vaccines made from mouse tissues. We hope to publish shortly our own experiments dealing with the immunization of ferrets with mouse vaccines and of mice with ferret vaccines. These, and our preliminary experiments on man to be mentioned shortly, indicate that no insuperable difficulties are likely to be met with on this account.

APPLICATION OF RESULTS TO VACCINATION OF HUMAN BEINGS.

Some facts make the outlook for the prophylactic vaccination of man against influenza appear gloomy; others make it more encouraging. For example, probably almost all human beings except very young children have some basic immunity to influenza. Any vaccine we inject will thus only be called upon to function as a secondary antigenic stimulus. We have previously shown that virus injected subcutaneously into ferrets with a waned immunity will give rise to more complete protection than when it is given to previously untreated ferrets (Smith, Andrewes and Laidlaw, 1935).

Francis and Magill (1936) have shown that several injections of living influenza virus cultivated on chick-embryo will cause a substantial rise in antibody content in human beings. We have for our preliminary experiments on man chosen to use virus from mouse lungs rather than culture-virus, because it will infect mice in such a high titre that we believe it to contain more virus particles than other products. We have, further, only used virus inactivated by formalin. It is true that living virus introduced subcutaneously into animals does not appear to multiply, and that Francis and Magill met with no untoward results after their human vaccinations with living virus. But a localization of virus in lung or nose may never happen when small numbers are tested under laboratory conditions, and yet the larger numbers of a field experiment may provide, now and again, the rare combination of circumstances necessary for infection to occur. Shope's (1936) experience with swine influenza is instructive. Living glycerolated virus given intramuscularly to pigs seemed in the laboratory to be an efficient and safe prophylactic; yet in the field it appeared that "two droves of swine . . .

probably acquired infections from virus used in the attempted immunization ". The use of living virus for immunizing man could be better justified were it not that our experiments indicate that inactivated virus may quite possibly be efficacious.

When mouse material is used for injection into man, it is necessary to be certain that no other viruses are present. We have worked with a stock of mice in which the virus of lymphocytic choriomeningitis (Armstrong and Lillie, 1934; Traub, 1935) has never been recognized. As an additional precaution all material to be worked up into vaccine has been tested by intracerebral inoculation into young mice. Dr. G. M. Findlay informs us in a personal communication that the choriomeningitis virus is rather more resistant to formaldehyde than that of influenza, requiring 10 days to be inactivated by 1 : 1000 formaldehyde in the cold. Although our vaccines have been filtered, we have added 1 : 10,000 merthiolate to them as an additional precaution against bacterial contamination; this strength of merthiolate appears to be harmless to the influenza virus as well as to man.

Preliminary tests of vaccine in man.—In preliminary tests on five subjects, we used virus which had been first washed and then formolized, in spite of the rather poor antigenic value of this product in mice. Later, we inoculated two men with formolized filtrate which had not been purified and gave each of them two doses a fortnight apart. In all cases 2 c.c. of vaccine were given into the left upper arm; the first two men (S. and A.) received the dose intramuscularly, the others subcutaneously.

The vaccine used for the first two volunteers caused a little temporary smarting which we believed to be due to free formaldehyde. Accordingly for later inoculations we adjusted the pH of the vaccine to 8 with dilute ammonia shortly before use, in order to convert any free formaldehyde to urotropin; the smarting was not wholly abolished by this procedure, but was not at all severe. The vaccines caused no general and only trifling local reactions. The two men (G. and E.) who received two inoculations of unpurified vaccine developed slight local œdema and tenderness after the second dose; doubtless they had been somewhat sensitized to mouse protein.

The effect of the inoculations on antibody-titre is shown in the table. Bleedings were made 10 to 14 days after inoculation. The titres are expressed in terms of the hyperimmune horse-serum (IH2) which we had used as our standard in previous studies of human sera (Andrewes, Laidlaw and Smith, 1935): thus S/5 implies a serum with one-fifth the potency of the standard serum. The sign <S/125 is equivalent to " no demonstrable antibody ".

Subject.	Vaccine.	Titre of serum. Before immunization.	After one dose.	After two doses.	Remarks.
S	1 dose washed vaccine	>S/125 <S/25	>S/25 <S/5	..	Increase in titre less than five-fold.
A	„ „	S/25	>S/25 <S/5	..	
T	„ „	<S/125	<S/25	..	Vaccine stored 40 days at −2° C. before use.
P	„ „	<S/125	S/25	..	
B	„ „	<S/125	S/5	..	
G	2 doses unwashed vaccine	<S/125	S/25	S/25	
E	„ „	>S/125 <S/25	>S/25 <S/5	S/5	

The table shows that the antibody-titre of the serum was definitely increased by vaccination in all the volunteers except T. Its extent was encouraging; some recent convalescents from influenza do not have serum titres better than S/25 to S/5. Rather to our surprise, the second dose of vaccine given to G. and E. caused no definite improvement on the result obtained by a single dose. The small numbers tested do not suffice to indicate whether the washed or unwashed vaccine will prove the better for human vaccination. In view of the results in mice, it is rather strange that a single dose of washed formolized virus gave as good results as it did. It is probable, however, that all the subjects tested had some basic immunity, even the four (T., P., B. and G.) who had no demonstrable antibodies in their serum before injection. Two of the men (P. and B.) were bled again 2½ months after vaccination; their antibodies remained at the raised level which was found shortly after vaccination. Further work is needed to show whether the increase in antibodies which follows vaccination of man will be reflected in increased resistance to infection with influenza.

SUMMARY.

1. Subcutaneous and intraperitoneal inoculation of living influenza virus into mice confers upon them substantial immunity against intranasal infection. Two doses confer much better immunity than one.

2. Influenza virus can be partially purified by washing on a collodion membrane without great loss of titre; this washed virus immunizes mice as effectively as does crude virus.

3. Influenza virus inactivated by 1 : 5000 or 1 : 10,000 formaldehyde immunizes mice almost or quite as effectively as does living virus. Virus inactivated by formaldehyde as strong as 1 : 1000 still makes a good vaccine. Formolized vaccine keeps its potency, at least in part, for 2 months in the cold.

4. Influenza virus which has been both purified by washing and inactivated by formaldehyde makes a relatively poor vaccine for mice.

5. Preliminary experiments on a few human beings indicate that subcutaneous inoculation of influenza virus inactivated with formaldehyde will produce in at least some subjects a substantial rise in their neutralizing antibodies for influenza virus.

6. The W.S. strain of influenza virus appears in the course of passages through mice to have undergone a rather sudden spontaneous change of such a nature that it is now possible to obtain filtrates of 10 to 100 times as high a titre as formerly.

We wish to thank our colleague, W. J. Elford, Ph.D., for kindly supplying us with gradocol membranes and assisting us with some of the washing experiments.

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AN IMPROVED PRECIPITATION REACTION VIEWING APPARATUS.

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THE present methods of examining a precipitate or ring opalescence in the precipitation reaction against a black background with oblique lighting are inadequate. The main objections to them are that in the majority there is a general light glare which interferes with the reading of very fine precipitates, while some require the removal of the tubes from their rack for examination.

We have designed a viewing apparatus which conforms to the following conditions :

1. It is capable of detecting the faintest trace of opalescence or flocculation, and differentiates easily between a simple opalescence and commencing flocculation.
2. The source of light is always invisible and cannot, therefore, interfere with the reading of the reaction.
3. No light is visible *unless there is a positive reaction.*
4. The intensity of the light is variable at will. By altering the light intensity the point of flocculation in an " optimal proportions " test is more easily determined.
5. The tube rack is an integral part of the apparatus so that the tubes are never moved during the experiment.
6. If the box is moved at all then all the tubes are moved equally.
7. The tubes fit the box exactly so that shaking is impossible once they are in place.
8. Extraneous light cannot interfere with the readings as the tubes are virtually in a miniature dark room.
9. It is never necessary to wipe the tubes during the course of an experiment. This is always done at the beginning before the tubes are placed in the box, after which they are removed from any possibility of contamination.

10. The tubes are close enough together to allow of the accurate comparison of any two, but are far enough apart for one not to interfere with the reading of another.

The apparatus was primarily designed for use in the precipitation reaction, and was demonstrated as such by us at the Laboratory Meeting of the Royal Society of Medicine (Section of Pathology) at University College Hospital in November, 1936. It was there suggested that it might equally well be applied to the detection of flocculation in the Kahn reaction, for comparing opacities or precipitates in protein determinations in the cerebro-spinal fluid, or for nephelometric work in general. It may, in fact, be used for examining for the presence of, or for the comparison of any opacities or precipitates in any fluid at room temperature.

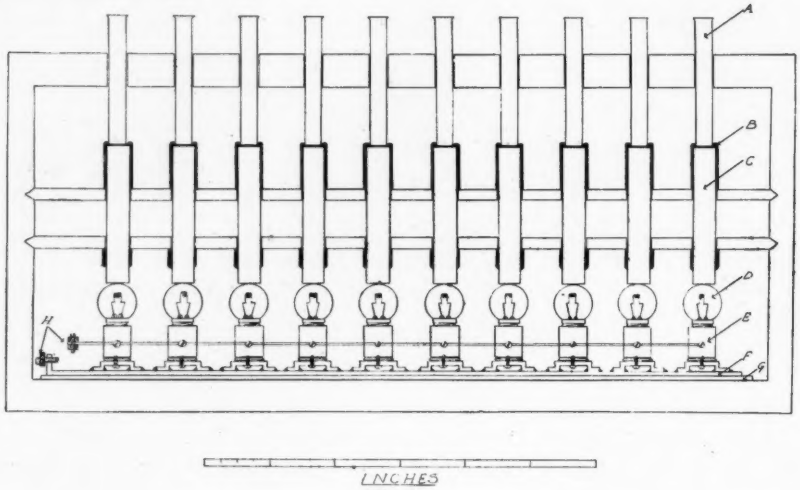

Fig. 1.

Description.

The apparatus is designed in a series of units, any number of which may be joined up as a whole to suit the requirements of individual workers. Our original box contained 10 such units, this number proving sufficient for most work.

Each unit (Fig. 1) consists of a 2 in. × $\frac{1}{4}$ in. flat-bottomed; clear glass agglutinating tube (A) which rests on a solid glass rod (C) 3 in. long and 9 mm. diameter, the ends of which have been ground square and finally polished, both the tube and rod being held in position by a black rubber collar (B). The lower end of the rod rests on a light unit consisting of a coil filament 8-volt 5-watt Osram light bulb (D) in a screw type bulb-holder (E). This lamp gives a beam of high light intensity from a small source.

It is essential in using this apparatus to see that all the tubes which are used are perfectly clean, both inside and outside. They are held by the top

end and wiped clean immediately before being placed in the box. The body of the tube must not be touched with the fingers after being wiped clean as even the natural sweat of the fingers gives a cloudy appearance to the tubes when the light is switched on.

The whole series of 10 units is enclosed in a compact wooden box, measuring 11 in. × 5 in. × 2 in. internally. The back opens along the bottom edge to allow any necessary adjustments or replacements and is made to be light-proof. The whole of the interior is painted a dull black (Fig. 2). The light units are mounted on a metal connecting plate (F) separated from the base of the box by an insulating plate (G), the lamp-holders being wired in parallel. The glass rods are held in position in 2 wooden racks by black rubber collars

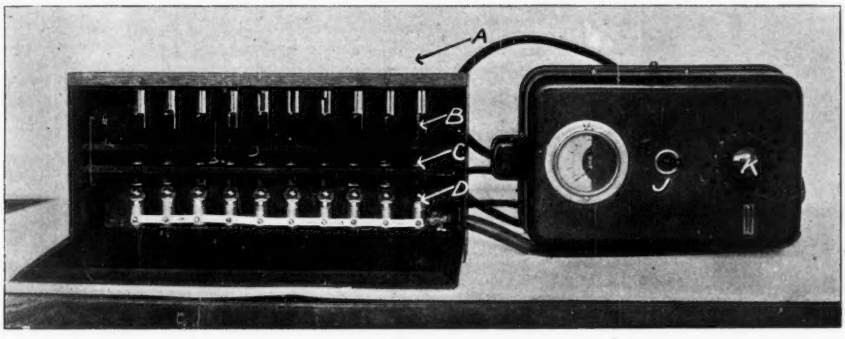

Fig. 2.

(B) above and below, the holes in the racks being only just large enough to allow the rods to pass through. These racks, which divide the box into two, are made to fit exactly so that no light from the lamp house below can reach the viewing chamber above except that which is transmitted through the glass rods.

The tubes are placed through holes in the top of the box, which are again cut exactly to size to prevent any lateral movement of the tubes, and rest upon the upper surface of the glass rods. The top rubber collar is cut so that it overlaps the glass rod and forms a foot in which the tube rests, at the same time preventing any light from being transmitted upwards outside the tube. When the collar is properly in position there can be no diffusion of light in the viewing chamber. The progress of the reaction is watched through slits $1\frac{1}{4}$ in. long and $\frac{1}{4}$ in. wide cut in the front of the box opposite the upper end of each unit. Through these slits the lower part of the tubes and the upper part of the black rubber caps alone are visible. The whole apparatus, opened from the back, is shown in Fig. 2. The lights are controlled through a variable A.C. mains transformer with a secondary output of 8 volts and 8 amps (K). The main switch is built in with the transformer.

The principle upon which the box is designed is that light is transmitted along a solid glass rod in parallel rays and is not scattered outside the walls

of the rod, the longer the rod the more parallel being the beam. We have found that the beam is sufficiently parallel for our purposes in a glass rod 3 in. long. The apparatus is so designed that a parallel beam of light of small area and high intensity is transmitted vertically upwards through the precipitin tube. If the tube is empty or filled with an optically clear fluid the light beam is not interrupted and continues in its upward path, no light being reflected forwards through the viewing slit and the tube appearing perfectly black (Fig. 3, Tube B). With fluids containing even very small amounts of particulate matter the Tyndall effect operates and the light beam is scattered in all directions by the particles in suspension, which appear through the viewing slit as a glowing ring.

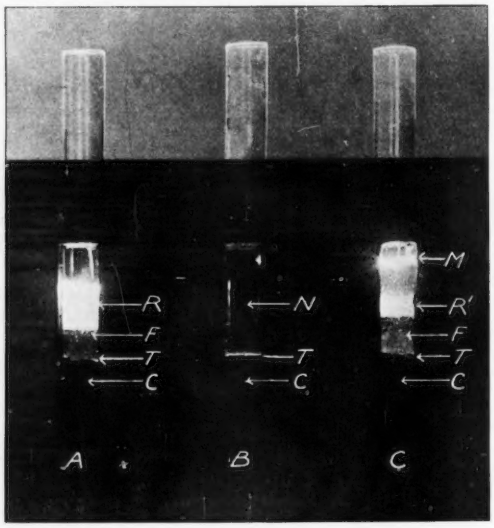

FIG 3.

The use of the box in the precipitation reaction is shown in Fig. 3, in which the tubes A and C show positive precipitates which were just visible to the unaided eye, while tube B is a negative control filled with clear serum. In tube A there is a heavy ring precipitate (R.) with marked flocculation (F.). No visible light reflection occurs at the bottom of the tube (T.), as this is prevented by the overlapping rubber cap (C.). The third tube, C, shows a lesser degree of ring formation (R′.) with commencing flocculation (F.). The reflection (M.) is that which occurs at the meniscus of the liquid in the tube. To prevent interference from this the meniscus should, as far as possible, be adjusted so that it is above the upper end of the viewing slit and therefore invisible. In practice it is found that the point of flocculation is more easily determined if the light intensity is lowered and then suddenly raised, as even the smallest floccules will glow if this is done.

Messrs. Charles Hearson have arranged to manufacture this apparatus to our specifications, which have been slightly modified for this purpose. Their standard box contains 12 units instead of 10, and the glass rods have optically polished ends, thus increasing the light intensity and reducing any diffraction at the ends. The rods are totally enclosed in a bakelite case, which serves the same purpose as the rubber cap in our original design and also prevents any loss of light intensity. They have arranged to make the box to take differing sizes of tubes so that it may be used for the variety of purposes indicated above.

EXPERIMENTAL RENAL LESIONS AND BLOOD-PRESSURE IN RABBITS.

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THE experimental proof of the occurrence of hypertension secondary to renal lesions has been a matter of considerable difficulty. Many workers have succeeded in raising the blood-pressure of experimental animals by means of direct attack upon the kidneys, whilst others, using the same or similar methods, have been unable to achieve this result.

Thus Katzenstein (1905), in the rabbit, and Goldblatt, Lynch, Hanzal and Summerville (1934), in the dog, have produced a rise in systolic pressure by compression of the renal artery, and Bell and Pedersen (1930–31) by incomplete obstruction of the renal vein. In the dog, Pässler and Heinike (1905), Janeway (1908–09, 1913) and Hartwich (1929–30) have all noted a rise in pressure following destruction of kidney substance, either by partial excision or by ligation of vessels. Cash (1924), however, found that this occurred only when some necrotic renal tissue was undergoing absorption within the body and when the total kidney substance was reduced by at least 50 p.c. The same result has also been achieved by Mosler (1912) on bilateral nephrectomy in cases in which rabbits survived this operation for more than 48 hours, by Bell and Pedersen on obstruction or ligation of the urethra, and by Rautenberg (1910) on intermittent obstruction of the ureter in the same animal. Hartman, Bolliger and Doub (1927) produced renal fibrosis in dogs by exposure of the renal area to high-voltage X-rays, and observed a gradual rise in pressure up to 40 mm. above the normal level in 14 days, accompanied by concentric hypertrophy of the left ventricle. Arnott and Kellar (1935) have produced a hypertension in rabbits by repeated intravenous injection of sodium oxalate. The rise in pressure occurred within 9 hours of the initial injection, and was greater when the animals had been previously fed on a " dry " diet in place of the usual cabbage diet. They have also found (1936) that denervation of

the kidney abolishes the hypertension of oxalate nephritis in unilaterally nephrectomized rabbits.

Other workers, however, have failed to produce hypertension by means of renal damage. Müller and Maas (1911) embolized the kidney extensively with paraffin without raising the blood-pressure, while Anderson (1926*a*) obtained in rabbits no rise on removal of one kidney and infarction or excision of up to half of the remaining organ, even in the presence of signs of severe renal insufficiency. The administration of a high protein diet to such animals (Anderson, 1926*b*) also had no pressor effect. In the same animal Dominguez (1928) carried out an extensive investigation of toxic conditions of the kidney following the administration of metallic poisons, such as lead, vanadium, uranium and radium, and produced all degrees of damage from acute swelling to granular contraction without change in the blood-pressure, whilst Backman (1914), Hartwich (1929–30) and Arnott and Kellar (1936) have shown that hypertension does not follow bilateral nephrectomy. These workers failed to produce hypertension, using the rabbit as the experimental animal and, in dogs, cats and rabbits Pearce (1909) and Miller and Miller (1911) failed to obtain pressor effects by the injection of extracts of kidney substance.

From these publications it would appear that in the dog many types of renal damage give rise to pressor effects, but that in the rabbit, the results obtained are much less conclusive.

METHODS.

Blood-pressure determinations were made by means of carotid loops prepared according to the method of van Leersum (1911). The operation employed was that described by Kremer, Wright and Scarff (1933), with the slight modifications that the infrahyoid muscles were not sutured behind the artery, and that continuous instead of interrupted silk sutures were used in the formation of the loop and for uniting the skin-edges of the neck. These sutures were easily removed about three weeks after operation, thus giving a softer and more pliable loop free from thickening due to retention of suture material. Anæsthesia was induced and maintained by means of open ether.

For recording the pressure the apparatus originally constructed by Scarff (1927) was employed, with the addition of a rigid backing to the bag, formed by two strips of wood hinged together at one end and provided with a clip at the other to retain the bag in position round the carotid loop. A system of air transmission of pressure from the bag to the surface of the mercury in the manometer was employed, which eliminated errors due to the relative positions of rabbit and manometer, and, by considerably reducing the inertia of the system, increased its sensitivity.

The blood-pressure was read by palpating the loop distal to the bag, and taking the average of the points at which the pulse disappeared and reappeared on raising and lowering the reservoir. This end-point was sharp, the figures differing generally by less than 2 millimetres. Six to eight readings were taken at each determination, and the last four were in very close agreement. Observations were made in the animal house at approximately the same time each day, thus ensuring standard conditions of environment and the minimum handling of the animals.

NORMAL BLOOD-PRESSURE IN THE RABBIT.

The arithmetical mean of all determinations made on normal rabbits was 96 mm., with a range from 74 to 120 mm., while 80 p.c. of the readings fell between 86 and 106 mm. These figures agree closely with those of Van Leersum (1911), Tigerstedt (1922), Koch and Koller (1932), Kremer, Wright and Scarff (1933), and Arnott and Keller (1935).

OXALATE NEPHRITIS.

In the first series of experiments blood-pressure determinations were made on rabbits which were receiving a course of intravenous injections of sodium oxalate solution.

The pathological changes in the kidneys following intravenous administration of oxalates have been known for many years. Kobert and Küssner (1879) observed the association of oliguria and heavy albuminuria with tubular degeneration and normal glomeruli, and described the formation in the tubules of calcium oxalate crystals, to the presence of which they ascribed the functional defect in the kidneys. Fraenkel (1880) and Mürset (1885) also described the occurrence of tubular degeneration, with oxalate deposits and granular and hyaline casts, but as some of their rabbits were passing excessive quantities of urine they considered that the effect of the oxalate crystals could not have been a simple mechanical blockage of the tubules. Dunn, Haworth and Jones (1924) carried out a detailed experimental study of the lesions in relation to their rapidity of occurrence, and to the amount and frequency of the oxalate dosage; while Browne and Dodds (1928) employed oxalate nephritis as the basis for an investigation of ante-partum hæmorrhage in relation to renal disease.

Six animals, all with carotid loops, were employed in the present investigation. Two of these were fed on an ordinary cabbage diet throughout and received no other preliminary treatment, whilst two, also on a cabbage diet, were subjected to unilateral nephrectomy 14 days before the commencement of the oxalate injections. The remaining two were placed upon a "dry" diet of bran, oats and bread, with greatly reduced fluid intake, the effect of which was to produce a urine which was acid in reaction and diminished in amount. On the 10th day unilateral nephrectomy was performed, and oxalate injections were commenced five days later.

A 1·34 p.c. solution of sodium oxalate was used, the dosage ranging from 3 to 7 c.c. intravenously, and containing from 41 to 94 mg. of the salt. This was given daily or at intervals of two days. Details of the dosage in the case of individual rabbits are set out in Table I. Death occurred in all 4 rabbits which received the maximum dose of 94 mg. of sodium oxalate, and was of a convulsive nature, occurring within a few minutes of the first or second injection of this larger dose, and therefore suggestive of tetany due to sudden lowering of the serum calcium. The remaining 2 rabbits, which each received 10 injections of 60 mg. of oxalate, showed no signs of tetany. The urine, however, was scanty in amount and contained heavy albumin, while the blood urea rose to 100 and 120 mg. respectively per 100 c.c., returning to normal about three weeks after cessation of the injections.

TABLE I.—*Details of Intravenous Oxalate Dosage.*

Rabbit number.	Preliminary treatment.	Number of injections.	Number of days.	Dosage: 1·34% solution.	Weight of oxalate.	Result.
488	..	7	9	3 × 3 c.c. 3 × 6 „ 1 × 7 „	456 mg.	Died, 9th day.
550	..	5	6	3 × 3 „ 2 × 6 „	281 „	„ 6th „
481	Nephrectomy	8	11	4 × 3 „ 2 × 6 „ 2 × 7 „	509 „	„ 11th „
482	„	8	11	4 × 3 „ 2 × 6 „ 2 × 7 „	509 „	„ 11th „
750	„ "Dry" diet	10	15	10 × 4·5 c.c.	606 „	Blood urea: 100 mg./100 c.c.
751	Do.	10	15	10 × 4·5 „	606 „	Blood urea: 120 mg./100 c.c.

In order to obtain an adequate control of the normal variations in pressure, blood-pressure readings were taken at intervals of 1 or 2 days for a period of 2 to 3 weeks prior to the commencement of the oxalate injections. In some cases the first few readings were low, due apparently to spasm of the artery on handling and insertion of the bag, or to fear or pain on the part of the rabbit, but satisfactory readings were soon obtained, and showed a constant average level for each rabbit, with a maximum fluctuation of about 10 mm. Observations were made at approximately the same time each day.

Daily readings of the pressure under standard conditions were made after the commencement of the oxalate injections, and in no case was any significant hypertension recorded. The highest pressure observed in any rabbit in this series was 110 mm., while the only effect of the oxalate appeared to be a slightly increased instability of the pressure, shown by a tendency to a greater swing above and below the normal average figure. Typical results from three rabbits, one from each group, are shown in Fig. I.

The four rabbits which died as the result of oxalate injections were subjected to post-mortem examination. In all cases the kidneys were enlarged and pale, while those in the rabbits which had had a preliminary nephrectomy were much increased in size. On section there was pallor of the cortex and swelling and congestion of the medulla. Sections stained with hæmatoxylin and eosin showed advanced tubular degeneration, with relatively intact glomeruli. The cells lining the convoluted tubules were swollen and necrotic, and in many cases contained large deposits of oxalate crystals, causing almost complete obliteration of the lumen of the tubule. Other tubules, both secreting and collecting, were swollen and contained massive hyaline and granular casts. There was no increase in the interstitial tissue. (See Plate.)

EMBOLIC GLOMERULAR LESIONS.

Simple Embolism.

In order to investigate the effect upon the blood-pressure of renal insufficiency due to mechanical glomerular obstructions without widespread renal infarction, a series of experiments was carried out in which embolic glomerular

lesions were produced. For this purpose the debris obtained from laked blood-corpuscles was injected directly into the renal artery of rabbits previously subjected to unilateral nephrectomy. This material (Baker, 1925) was selected as being fine enough to pass through the arterial system of the kidney without producing massive infarction of the organ, and yet on account of its viscosity and adhesive tendencies likely to be held up in the glomerular tufts. Ten c.c. of oxalated rabbit's blood were centrifuged for 30 minutes at 3000 r.p.m., and the cells thus obtained were washed three times by suspension in normal saline and subsequent centrifugalization. 0·5 to 1 c.c. of distilled water was then added to the washed cells to produce laking, and the tube was placed in the ice-chest overnight. The resulting debris was sufficiently viscid to pass with difficulty through a fine intradermal needle.

In each case right nephrectomy was first performed and the wound allowed to heal. The rabbit's left kidney was then exposed through the usual lumbar incision, and the kidney delivered into the wound. 0·5 c.c. of debris was injected directly into the renal artery through a finely-ground needle, the circulation being intentionally obstructed for a few seconds after the termination of the injection. The kidney was returned to the abdomen, and the wound closed. When a fine enough needle was used subsequent hæmorrhage from the renal artery rarely occurred. After injection blood-pressure readings and blood-urea determinations were made at regular intervals.

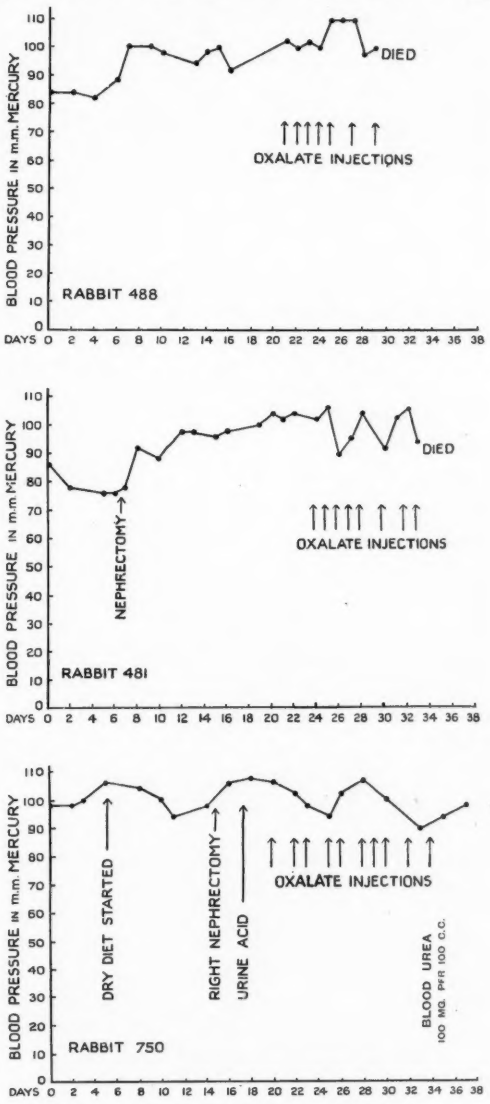


FIG. I.—Blood pressure in oxalate nephritis.

Seven rabbits survived the initial operation of injection of the renal artery. Five of these received a second injection of laked blood debris at a later date, while in one animal it was possible to inject the renal artery on three occasions. By this means a moderate degree of renal insufficiency was induced, as shown by a rise in the blood-urea from a normal level of about 30 mg. per 100 c.c. to levels of 80, 100, or, in one instance, 180 mg. per 100 c.c. The maximum blood-urea readings were obtained in all cases within 4 days of injection, after which there was a gradual decline, until by the end of a further week or 10 days the blood urea had regained, or almost regained, the normal level. A similar or greater rise in the blood urea then occurred on subsequent injection.

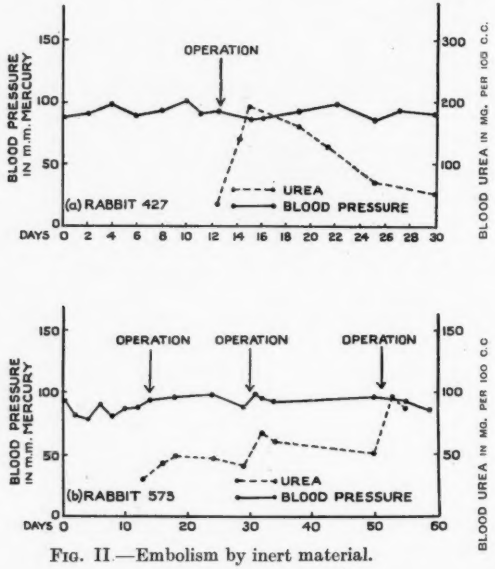


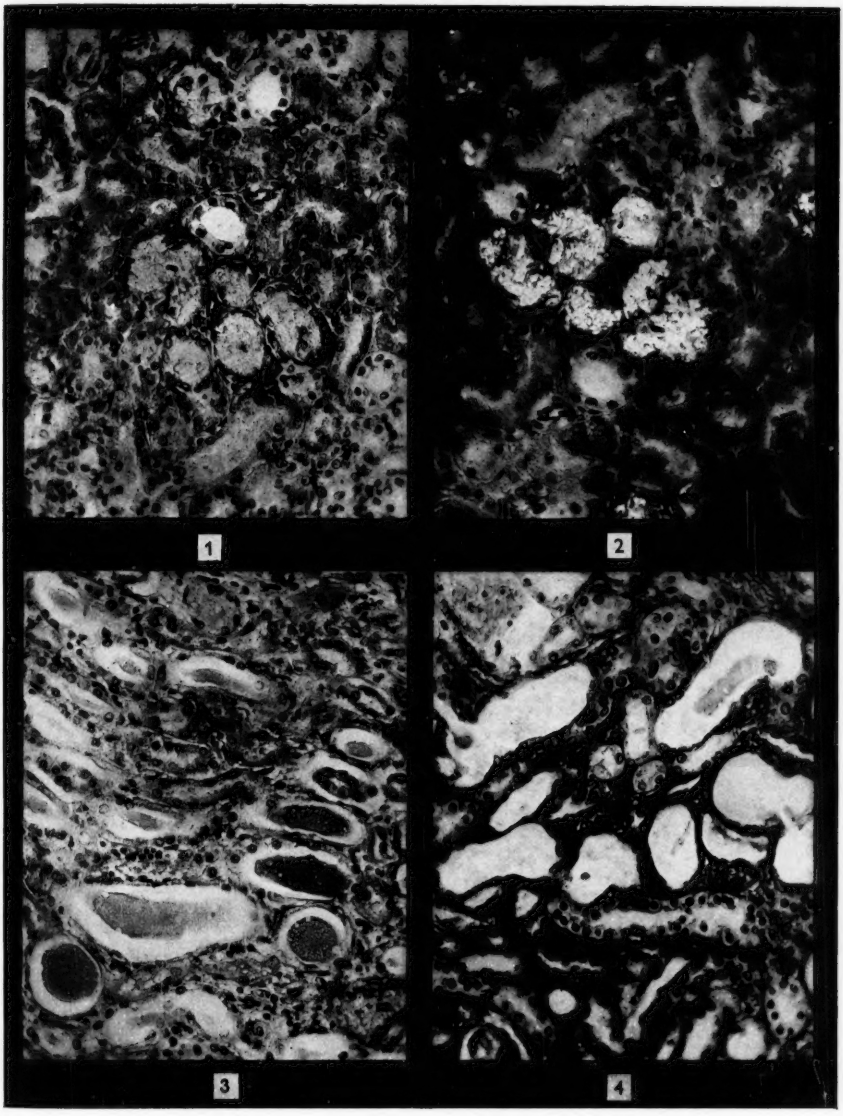
FIG. II.—Embolism by inert material.

The blood-pressure of each rabbit was carefully standardized over a period of at least two weeks before operation, and in no case did any significant increase occur during the periods of induced renal insufficiency. Typical results obtained from rabbits receiving one and three injections respectively are shown in Fig. II.

In one case the remaining kidney was then removed, and in another the ureter was ligated. Both animals died on the third day, with blood-urea readings of 280 and 380 respectively, but without alteration in the height of the blood-pressure.

Killed Streptococci in Sensitized Rabbits.

With the recognition of the frequent association of acute nephritis with bacterial infections, especially those of the streptococcal type, numerous attempts have been made to reproduce in animals the glomerular lesion typical



Figs. 1 and 2.—Oxalate deposits. Uncrossed and partly crossed Nicol prisms.

Figs. 3 and 4.—More advanced tubular degeneration.

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of the disease in man. The results of the earlier experiments with various types of organism in normal animals were disappointing, but the use of sensitized animals has given more encouraging results. MacNider (1924) and Leiter (1924) have reviewed the literature of the earlier attempts to produce acute and chronic lesions respectively, while the more recent work has been reviewed by Longcope and Lukens (1931). The latter workers by injecting heat-killed hæmolytic streptococci into the renal artery of rabbits produced glomerular lesions in half the animals, and especially in those in which an acute localized infection had been previously induced by intradermal inoculation of the living organism.

In the present investigation an attempt was made to produce glomerular lesions by injection of heat-killed hæmolytic streptococci into the renal artery of previously sensitized rabbits. Five strains of hæmolytic streptococci were obtained from the throats of patients suffering from scarlet fever, and living cultures of these organisms were injected intradermally into rabbits, each animal receiving three injections at intervals of one week. The first injection in each case produced an acute cutaneous inflammatory lesion, while subsequent injections were followed by lesions of much less severity. Two weeks after the third injection the right kidney from each animal was removed, and 0·5 c.c. of a heavy suspension of heat-killed streptococci of the appropriate strain injected into the left renal artery.

The blood-pressure and blood-urea findings in these animals are given in Table II.

TABLE II.—*Killed Streptococci in Sensitized Rabbits.*

Rabbit number.	Previous readings.		Days after injection.					
	Range.	Average.	3.	9.	14.	27.	40.	100.
7	**104–88**	**94**	**94**	**88**	**90***	**92**	**108**	**104**
	..	..	(45)	(67)	(67)	(55)	(37)	(57)
788	**116–100**	**108**	**104**	**86**	**90*†**	..	..	..
	..	..	(102)	(79)	(77)	..	..	..
749	**120–98**	**108**	**136**	**120**	**122**	**112**	**102**	**120**
	..	..	(168)	(57)	(56)	(52)	(37)	(50)
748	**104–96**	**100**	**118**	**118**	**110**	**104**	**108**	**104**
	..	..	(38)	(52)	(64)	(59)	(42)	(45)
778	**86–70**	**80**	**94**	**86**	**92**	**92**	**90**	**110**
	..	..	(39)	(59)	(71)	(54)	(50)	(40)

Figures in heavy type = blood-pressure in mm. of mercury. Figures in light type = blood-urea in mg. per 100 c.c.

* Second injection 14th day.

† Death on 16th day.

With one exception (Rabbit 749) there was no appreciable rise in the blood-pressure, and in that one instance the increase was slight and of short duration.

A second injection of the appropriate organism was attempted in Rabbits 7 and 788 on the 14th day, but the latter animal died 2 days later from intercurrent infection. Microscopic section of the kidney of this animal showed characteristic glomerular lesions as described by other workers. The

histological changes produced by this method are still being investigated and will not be described here.

SUMMARY.

Experiments are described in which blood-pressure readings have been taken in rabbits after various forms of renal injury; these injuries include oxalate nephritis, unilateral and bilateral nephrectomy, trauma, ligation of ureters, glomerular embolism both by inert material and by killed bacteria in sensitized animals.

In many of the animals considerable impairment in renal function was indicated by an increase in the blood urea, but in none of them was there found any significant rise in the blood-pressure.

Our thanks are due to Prof. McIntosh, Director of the Bland-Sutton Institute of Pathology, for much help in this work, and to Prof. E. C. Dodds, of the Courtauld Institute of Biochemistry, for the chemical findings.

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THE ANTIGENIC COMPOSITION AND VIRULENCE OF *BACT. TYPHOSUM* GROWN ON A CHEMICALLY-DEFINED MEDIUM.

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In a summary of the knowledge relating bacterial antigenic composition to nutritional requirements, Braun (1936) has suggested that bacteria possess three degrees of potentiality for antigenic synthesis: (1) Ability to synthesize a particular antigen quite independent of the medium on which the organism is grown. In this group he has included those strains of *Bact. typhosum* in which the Vi antigen appears to be stable on nutrient agar even on repeated subculture. (2) Ability of synthesis which is dependent on the conditions of growth. As an example he quoted the necessity of a complex (laboratory) medium for the development of flagella of the typhoid-coli group; also those V strains of *Bact. typhosum* which need ascitic fluid or serum for development of the Vi antigen (Kauffmann, 1935*a*). (3) Cases in which the ability to produce a particular antigen has been lost or never developed, and in which this cannot be regained under optimal conditions of growth. This group would include strains of a normally flagellated species which had no flagellar antigen and strains of *Bact. typhosum* which are devoid of Vi antigen. Kauffmann (1935*a*), however, found that all strains of *Bact. typhosum* when freshly isolated from the body possess Vi antigen—a finding which has been confirmed by Felix, Krikorian and Reitler (1935).

A direct correlation between the growth requirements of an organism and its virulence can only be satisfactorily obtained in a medium whose chemical composition is known. Does the transference of an organism to such a medium affect its antigenic structure and virulence? On this question there is some conflict of evidence. Seibert, Long and Morley (1933) stated that they were able to convert a smooth avian tubercle bacillus into its rough variant by growth in Long's synthetic medium. On the other hand, Petroff (quoted by Steenken, 1935) claimed to have dissociated a virulent variant from the B.C.G. strain of *Myco. tuberculosis* by growth in Proskauer and Beck's synthetic medium.

The most comprehensive work on the subject is that of Braun and Cahn-Bronner (1921*a*, *b*; 1922). They (1921*a*) grew *Bact. typhi murium* on a synthetic medium in which the sole source of carbon and nitrogen was ammonium lactate. Apart from some loss of the flagellar antigen, antigenic composition was unaltered. They further showed that the organism was still virulent for mice after several subcultures on this simple medium. In a later paper (1922) a similar result was obtained with three strains of *Bact. paratyphosum B* and

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two strains of *Bact. enteritidis* (Gärtner). Five ammonia-assimilating strains and three ammonia non-assimilating (exacting) strains of *Bact. typhosum* were also used. None of these was pathogenic for mice, even when grown in broth. As in the case of *Bact. typhi murium* and the other members of the Salmonella group, the only serological difference observed when the organism was cultivated in simple media of known composition was some loss of the flagellar antigen.

The present paper amplifies the work of Braun and Cahn-Bronner. The strains of *Bact. typhosum* originally described by Felix and his co-workers (Felix and Pitt, 1934*a*, *b*; 1935; Felix, Bhatnagar and Pitt, 1934; Felix, Krikorian and Reitler, 1935; Felix and Bhatnagar, 1935) have been used. Unlike the strains used by Braun and Cahn-Bronner, these strains possess well-defined virulence for the mouse. Associated with this virulence is the presence of the somewhat specialized Vi antigen, and living suspensions of these strains also possess the property of being inagglutinable with a pure somatic (O) antiserum prepared from strains not possessing the Vi antigen. This work, although challenged by Tao (1936), who did not take the precaution to use monospecific antisera, has been confirmed by Perry, Findlay and Bensted (1934), Kauffmann (1935*a*, *b*), Dyachenko (1936), Horgan (1936), Craigie and Brandon (1936*a*, *b*) and others.

The method used in the present work has been to grow these strains on a variety of simple, chemically-defined media, and then to investigate any change in virulence and agglutination reactions that might have occurred. In the section headed "Experimental" the first part is concerned with the effect of the nitrogen sources, with special reference to amino-acid nutrition; the second part deals with the effect of the carbon and/or energy sources.

TECHNIQUE.

Strains.

Four strains of *Bact. typhosum* were used (for details see Felix and Pitt, 1934*a*): H901 possessing H and O but not Vi antigens; O901 possessing O but not H or Vi antigens; "Watson", possessing all three antigens; Ty2, also possessing all three antigens.

The terminology of Kauffmann (1935*a*) has been followed: strains possessing the Vi antigen are referred to as V strains; those in which this antigen is absent, as W strains. V strains are not agglutinated by a pure O antiserum; W strains are. The strains were kept on nutrient agar and subcultured two or three times per week. They were plated out periodically and single smooth colonies selected. The V strains were tested periodically for agglutination with an O antiserum. Most of the work has been undertaken with strain Ty2. This is a very stable strain, which shows little tendency to lose its Vi antigen or become O-agglutinable under laboratory conditions.

Agglutination Reactions.

Antisera.

Mono-specific sera were used as follows:

Pure O antiserum prepared by three weekly injections of strain O901 heated to 55° C. for 1 hour.

Pure H antiserum supplied by Dr. Felix. A serum prepared against a motile rough strain of H901.

Pure Vi antiserum. A serum prepared against a V strain of *Bact. typhosum* was supplied by Dr. Hartley. This serum, which contained antibodies against all three antigens (H, O and Vi), was absorbed three times with a massive dose of strain H901, until no agglutination was given with either strain H901 or O901 in a dilution of 1/80. As mentioned by Felix (Felix, Bhatnagar and Pitt, 1934), the titre varied slightly with the particular V strain used.

Suspensions.

Except where otherwise stated, the suspensions used for agglutination were prepared as follows: Living 18-hour cultures in synthetic medium were centrifuged and the organisms suspended in normal saline. Each suspension was standardized by the opacity method to 400×10^6 organisms per 1 c.c. Readings were taken after 2 hours in the water-bath at 37° C. and then overnight at room temperature.

Virulence Tests.

Initial experiments were carried out with mice of weight 25–30 g. Later it was found that mice varying in weight from 20–35 g. were all equally susceptible to infection. The experiments were controlled with mice of the same weights inoculated from a culture grown on an agar slope. Albinos were excluded because of their alleged resistance to infection with Salmonella organisms (Pritchett, 1926).

Inoculation was made intraperitoneally. The liquid cultures were centrifuged and the organisms suspended in Ringer's solution. The suspension was standardized by the opacity method (in many cases a viable count was made on the inoculum at the same time) and 0·5 c.c. was injected. As a routine two doses (100×10^6 and 200×10^6 organisms) were used, and these were injected into groups of 10, 6 or 4 mice. Each experiment was watched for at least one week. Nearly all deaths occurred on the first 2 days after inoculation. A post-mortem was carried out on all dead mice, the organisms being recovered from the heart's blood and recognized by agglutination with a specific serum.

Growth Tests.

Media.

The following synthetic media were used:

Amino-acid medium resembled that used by Fildes *et al.* (1936) for the growth of staphylococcus. It contained 16 amino-acids as follows:

*s-alanine, s-valine, s-leucine, s-glycine, *l*-proline, *l*-oxyproline and s-aspartic and *d*-glutamic acids . . .	*M*/1500†
s-methionine, s-phenylalanine, *l*-tyrosine, *l*- arginine, *l*-histidine, *l*-lysine and *l*-cystine	*M*/4000
dl-tryptophan	*M*/20,000

* s- denotes an amino-acid of synthetic origin.

† Representing molarities of one only of the optical isomers of racemic mixtures.

together with phosphate buffer as follows:

KH_2PO_4	$M/30$
$N/1$ NaOH	26 c.c. per litre (to give pH of 7·6)

With the exception of tryptophan and cystine, quantities required for 1 litre of complete medium were dissolved in 600 c.c. and the pH adjusted to 7·6. The medium was tubed in 6 c.c. quantities in Monax test-tubes 6 × $\frac{3}{4}$ in. and autoclaved at 115° C. for 20 minutes. The following solutions were added to each tube with sterile precautions:

‡$M/80$ cystine in $N/10$ HCl	0·2 c.c.
‡$M/200$ tryptophan	0·1 c.c.
‡$M/60$ $MgSO_4$, $7H_2O$	0·1 c.c.
§$M/2$ $NaHCO_3$	0·5 c.c.
§$M/2$ glucose	0·25 c.c.
(*or* ‡$M/2$ sodium lactate	0·5 c.c.‖)
Distilled water to	10 c.c.

‡ Sterilized separately by autoclaving at 115° C. for 20 minutes.
§ Sterilized by filtration through a glass Seitz filter (Knight and Fildes, 1936).
‖ Since one molecule of glucose was equivalent to two molecules of lactate, double the quantity of sodium lactate was added.

Ammonia medium.—The constituents were as follows:

KH_2PO_4	4·5 g. ($M/30$)
$N/1$ NaOH	26 c.c.
NH_4Cl	0·5 g. ($M/108$)
$(NH_4)_2SO_4$	0·5 g. ($M/264$)

These quantities, required to give one litre of medium, were dissolved in 600 c.c. of distilled water. The medium was tubed in 6 c.c. quantities and autoclaved at 115° C. for 20 minutes. To each tube was added the following sterile solutions:

‡$M/60$ $MgSO_4$, $7H_2O$	0·1 c.c.
§$M/2$ glucose	0·25 c.c.
(*or* ‡$M/2$ sodium lactate	0·5 c.c.‖)
§$M/2$ $NaHCO_3$	0·5 c.c.
Distilled water to	10 c.c.

‡ Sterilized separately by autoclaving at 115° C. for 20 minutes.
§ Sterilized by filtration through a glass Seitz filter (Knight and Fildes, 1936).
‖ Since one molecule of glucose was equivalent to two molecules of lactate, double the quantity of sodium lactate was added.

Purity of chemicals.

All substances were of the finest commercial grade and, except for sodium bicarbonate, were further re-crystallized several times.

pH.

The pH of each basal medium, after autoclaving, was 7·6. On the addition of bicarbonate, this rose to 8·0. The final adjustment to 7·6 was effected by the conditions of incubation (see below).

Incubation.

Cultures were incubated at 37° C. in an atmosphere of air containing 5 p.c. of carbon dioxide (Gladstone, Fildes and Richardson, 1935). The tubes were placed in large screw-capped jars. Into the lid of each was soldered a copper tube, by which the jar could be evacuated and refilled with air containing 5 p.c. of carbon dioxide.

Inoculum.

From a solid culture, a just-visible suspension was made in distilled water and 3 drops inoculated per tube; when subculturing into the same medium as that of the inoculum, 3 drops of the undiluted liquid culture were used per 10 c.c. of medium.

Amount of growth.

This was roughly estimated by the opacity method.

EXPERIMENTAL.

Van Loghem (1911) and Braun and Cahn-Bronner (1921*b*, 1922) showed that, with regard to nutritional requirements, strains of *Bact. typhosum* fall into two groups—exacting and non-exacting. The former require to be given for growth a number of amino-acids, of which tryptophan is the most important. Organisms of the second group are able to synthesize these acids for themselves from a simple nitrogen source, such as ammonia. They are, therefore, able to grow in a medium containing ammonia as the sole nitrogen compound present. In our work all four strains examined were found to be exacting—that is, they were not able to grow directly on ammonia as the sole nitrogen source. It is possible by a process of "training" to convert an exacting into a non-exacting strain (Fildes, Gladstone and Knight, 1933). This is accomplished by subculturing the organism into media containing successively fewer amino-acids, the nitrogen content being maintained by the addition of ammonia. It was decided to investigate how far this process affected the virulence and antigenic composition of the organism.

Effect of the Nitrogen Source on Virulence and Antigenic Composition.

Growth in the amino-acid-glucose medium.

When cultured on the amino-acid-glucose medium, inoculated direct from agar, all four strains grew equally well and could be subcultured indefinitely. After it had grown for a number of subcultures on this medium, the virulence and agglutination reactions of strain Ty2 were tested (Table I). Even after 46 subcultures on the amino-acid-glucose medium, the organism has remained fully virulent and no alteration in the agglutination reactions with pure O and Vi antisera has taken place.

The need for amino-acids other than tryptophan, and perhaps cystine, disappears in the course of a very few subcultures from the amino-acid

TABLE I.—*Agglutination Reactions and Virulence of Strain Ty2 after Growing in the Amino-acid-glucose Medium.*

Agglutination Reactions.

Medium	Amino-acid-glucose.				Nutrient agar.	
Agglutination with	O antiserum.		Vi antiserum.		O antiserum.	Vi antiserum.
Number of subcultures	6	46	6	46		
Serum dilutions:						
1 : 400	0	0	+++	+++	0	+++
1 : 800	0	0	++	++	0	++
1 : 1600	0	0	+−	+	0	+−
1 : 3200	0	0	tr.	tr.	0	tr.
1 : 6400	0	0	0	0	0	0
1 : 12,800	0	0	0	0	0	0
0·9 p.c. saline	0	0	0	0	0	0

Virulence Tests.

Medium.	Number of subculture.	Number of organisms injected ($\times 10^6$).	Number of mice injected.	Number of specific deaths.
Amino-acid-glucose	6	100	10	10
	46	100	6	5
		200	6	6
Nutrient agar	..	100	10	9

Note.—Plus signs refer to the degree of agglutination: +++ complete, ++ almost complete, + standard, +− less than standard, tr. trace.

medium; indeed the organism will usually grow in one subculture upon tryptophan, cystine, ammonia and glucose, if a large inoculum from the complete amino-acid-glucose medium is used. Growth may, however, be somewhat delayed.

Both V strains (Ty2 and "Watson") were subcultured 6 times on a tryptophan-ammonia-glucose medium and tested for virulence and agglutination reactions. There was no agglutination with a pure O antiserum in a dilution of 1/400. Strain Ty2 agglutinated with a pure Vi antiserum to a titre of 1/1600, the strain "Watson" somewhat higher. The organism was still virulent, killing all of a group of ten mice in a dose of 100×10^6.

The removal of tryptophan: growth in the ammonia-glucose medium.

It is possible moreover to remove tryptophan from the medium and grow the organism in ammonia as the sole nitrogen source. This can be done in the following ways:

Method 1.—The most successful method of training the organism to utilize ammonia was to grow it in a mixture of those amino-acids (alanine, valine, leucine, arginine, aspartic acid, methionine, phenylalanine and oxyproline) which it could be most readily trained to do without. These provided a variety of nitrogenous material from which tryptophan could be synthesized. To them were added an ammonium salt, glucose and the smallest amount of tryptophan ($2 \times 10^{-7}M$) which would just support growth. After a slight delay the organism then grew heavily. Thereafter all amino-acids could be

omitted from the medium and growth proceeded on ammonia as the sole nitrogen source. Training by this method was very rapid.

The virulence and agglutination reactions of strain Ty2, trained in this way to assimilate ammonia, were now tested. After seven subcultures no alteration in virulence and agglutination reactions with pure O and pure Vi antisera had occurred. A dose of 100×10^6 organisms killed 8 out of 10 mice; an agar-grown culture, 7 out of 10. After 27 subcultures, agglutination by a pure O antiserum in a dilution of 1/400 was just detectable with a lens. Vi-agglutination, however, was unaltered, and virulence was the same as in an agar-grown control. By plating out on the ammonia medium solidified by the addition of 2 p.c. alcohol-precipitated bacto-agar and selecting colonies which showed no O-agglutination, the resistance to agglutination with an O antiserum of the trained strain was maintained.

Nine cultures from the same strain, trained to grow in this way on ammonia, all agglutinated completely with Vi antiserum and 8 gave no agglutination with a pure O antiserum after a number of subcultures on the ammonium medium; one gave partial agglutination with an O antiserum, but no difference in virulence between this and other strains was found. It is thus seen that virulence was unaffected by wide changes in the sources of nitrogen which the organism could be trained to use.

Method 2.—Another method of training the organism to dispense with tryptophan and to utilize ammonia was also used. This was similar to that of Braun and Cahn-Bronner (1922), and consisted in prolonged incubation in a medium containing ammonia, glucose and the minimum amount of tryptophan which just allowed visible growth. Of the four strains treated in this way only one, a strain not possessing the Vi antigen, became adapted to grow on ammonia after 6 days' incubation. There was no increase in growth in the other strains beyond the trace observable on the first day, and no growth occurred when this was subcultured into the ammonia medium. One of the V strains (Ty2) was inoculated, after 6 days' incubation in this inadequate medium, into the same medium *plus* alanine, valine, leucine, arginine, aspartic acid, methionine, phenylalanine and oxyproline as in the previous method of training. After 2 days, growth occurred and the organism could be subcultured indefinitely on the ammonia medium.

After 6 subcultures on this medium it was tested for virulence and agglutination reactions. It was found that it had completely lost its Vi antigen and was agglutinated to titre by an O antiserum. On injection into groups of 4 mice in three separate doses of 400×10^6, 200×10^6 and 100×10^6, not a single mouse died. There was, therefore, complete loss of virulence and Vi antigen. This result is discussed later.

Test for roughness.—On plating this strain out on nutrient agar, all the colonies appeared smooth and the culture was not agglutinated by an antiserum prepared against a rough strain of *Bact. typhi murium*. After 40 subcultures on the ammonia-glucose medium, about 50 p.c. of the colonies appeared rough. This culture was stable to 2 p.c. saline, but was agglutinated by the rough *Bact. typhi murium* antiserum. On absorption of the serum in a dilution of 1 : 50 with $36{,}000 \times 10^6$ organisms of this mixed strain from the ammonia-glucose medium, the titre for its homologous organism was reduced from 1 : 6400 to 1 : 200. Absorption with the same number of organisms from a strain which had retained its virulence and Vi antigen, after being adapted to utilize ammonia and grown for the same number of subcultures on the

ammonia-glucose medium, gave no reduction in titre. It appears therefore that the organism, once it had lost its Vi antigen and had become agglutinable with an O antiserum, tended to become rough. Felix (Felix and Pitt, 1935) and Kauffmann (1935*a*), however, have described certain strains which retain their Vi antigen even after they have become rough.

Attempt to recover virulence and Vi antigen.—Perry, Findlay and Bensted (1934) were able, by rapid passage through mice, to " rejuvenate " the Rawlings strain of *Bact. typhosum* so that it became highly virulent for the mouse. It did not lose its rough colonial appearance, but became stable to 6·4 p.c. saline and resembled the strains of Felix containing Vi antigen (V strains of Kauffmann) in the poor agglutination of living cultures with a pure O antiserum.

We have not been successful in causing our strains to regain virulence by mouse passage. After 13 serial passages, the ammonia-assimilating strain which had lost its virulence remained avirulent, still agglutinating to titre with an O antiserum and not being agglutinated by a pure Vi antiserum. Ability to grow in the ammonia medium was not lost by such passage.

Attempts were also made by rapid subculture in the amino-acid-glucose medium. After 55 daily subcultures, the organism was still agglutinated by a pure O antiserum, and remained avirulent and inagglutinable with a pure Vi antiserum. When inoculated back again into the ammonia medium growth was still obtained, showing that the organism still retained its characteristic synthetic ability with regard to nitrogen assimilation.

The effect of growth on simple media of known composition on the production of the flagellar antigen.

Braun and Cahn-Bronner (1921*a*, *b* ; 1922) showed that some loss of the flagellar antigen of *Bact. typhi murium*, *Bact. paratyphosum B*, *Bact. enteritidis* (Gärtner) and *Bact. typhosum* occurred when these organisms were grown in synthetic media. In the case of *Bact. typhosum* we have been able to confirm this work. The strain Ty2 which is actively motile on nutrient agar became almost completely non-motile in the amino-acid medium. When trained to grow on ammonia, motility was lost completely.

By absorption of a pure H antiserum with a standard number of organisms it has been possible to investigate the amount of flagellar antigen present (Table II).

In Table II it is seen that, although a small amount of flagellar antigen was present in organisms grown in the amino-acid medium, it had completely disappeared when the organisms were trained to grow on ammonia. The flagellar antigen, however, is only lost temporarily, and can be completely restored when the organism is transferred to nutrient agar.

The Effect of the Carbon Source on Maintenance of Virulence and Persistence of the Vi Antigen.

All experiments so far recorded were done with glucose as the main source of carbon and energy. In order to investigate the effect of alteration in the carbon sources on the agglutination reactions and virulence, the glucose in the ammonia medium was replaced by lactate. This had a considerable effect

TABLE II.—*Test for Residual H Agglutinins after Absorption of a Pure H Antiserum with Organisms of Strain Ty2 Grown in Chemically-defined Media.*

Agglutination of an H Typhoid Suspension with Pure H Antiserum.

Serum dilutions.	Serum absorbed with 20,000 × 10^6 organisms grown on—				
	Agar.	Amino-acid glucose.	Ammonia-tryptophan-glucose.	Ammonia-glucose.	Unabsorbed serum.
1 : 160	+	+++	+++	+++	+++
1 : 320	tr.	+++	+++	+++	+++
1 : 640	0	+++	+++	+++	+++
1 : 1280	0	+++	+++	+++	+++
1 : 2560	0	+++	+++	+++	+++
1 : 5120	0	++	+++	+++	+++
1 : 10,240	0	+	+++	+++	+++
1 : 20,480	0	tr.	++	+++	+++
1 : 40,960	0	0	+	++	++
1 : 81,920	0	0	tr.	+	+
1 : 163,840	0	0	0	tr.	tr.
1 : 327,680	0	0	0	0	0

Note.—Absorbing suspensions: Liquid cultures centrifuged and organisms suspended in normal saline. Suspensions heated to 60° C. for 1 hour and standardized by the opacity method. Serum absorbed in a dilution of 1 : 80 for 4 hours at 37° C., and then overnight in the ice-chest.

Agglutinating suspension: Strain Ty2 grown in Hartley's nutrient broth for 18 hours, formolized, heated to 60° C. for 1 hour and standardized by the opacity method to 500 × 10^6 organisms per 1 c.c. Agglutination reaction carried out at 56° C.

on growth. Growth in 18 hours in 10 c.c. of medium in an upright tube was very poor and was not greatly increased by longer incubation. Thus, in order to obtain the standard of opacity for testing the agglutination reactions, several tubes of medium were necessary. When tested against a pure O antiserum, the strain Ty2 grown in the lactate medium agglutinated to a titre of 1/3200. When it was grown on glucose medium, on the other hand, only a faint trace of agglutination in a dilution of 1/400 was apparent. With a pure Vi antiserum, little difference was noted between glucose and lactate cultures; if anything, the latter was agglutinated to a slightly higher titre than the former. Growing on lactate, therefore, the organism has lost its resistance to an O antiserum. Owing to the very poor growth, however, the conclusion that the effect is due to the utilization of lactate for growth in place of glucose is hardly justified.

The effect of aëration.

In order to increase the growth on the tubes containing lactate, air containing 5 p.c. CO_2 was passed continuously through the medium. A control containing glucose in place of lactate as the source of carbon was similarly aërated. The growth in both cultures was found to be much increased by aëration. This was particularly the case in the lactate cultures, so that in 24 hours the growth was nearly equal in the two tubes. Subcultures were made every 24 hours except over the week-end, when the cultures were placed in the ice-chest. A parallel series was made in stagnant tubes. On testing the agglutination reactions of the 3rd, 7th and 10th subcultures, all lactate cultures agglutinated with an O antiserum to a titre of 1/3200. The stagnant glucose cultures remained inagglutinable with an O antiserum, but the aërated

glucose cultures somewhat unexpectedly became progressively less and less resistant, until at the 10th subculture both glucose and lactate cultures agglutinated to the same titre with a pure O antiserum. Vi-agglutination showed no change, except that stagnant glucose cultures agglutinated to a somewhat lower titre. All cultures were equally virulent, and showed no difference from a strain grown on agar.

On plating out the 10th subculture on to nutrient agar, two types of colonies were obtained from the aërated cultures and from the stagnant lactate cultures, differing in opacity under oblique illumination. Several colonies of each kind were picked off and tested against pure O and Vi antisera. The more opaque variety agglutinated to titre with a pure Vi antiserum, but showed no agglutination with a pure O antiserum. The more transparent were for the most part not agglutinated by the Vi antiserum, but were completely agglutinated by the O antiserum. In some cases partial agglutination with a Vi antiserum was observed, which was lost on replating and reselecting colonies. This difference in colonial appearance between strains containing Vi antigen (V strains) and those that have lost this (W strains) has also been described by Craigie and Brandon (1936*a*); this finding also supports their contention that strains showing agglutination with both Vi and O antisera (Kauffmann's VW strains) are in reality mixtures of V and W forms. The stagnant glucose cultures gave rise to the V forms only. In spite of the appearance of W forms in the aërated glucose and lactate cultures, the fact that Vi agglutination of the cultures as a whole showed little change and the culture was still virulent suggested that there were still present a large number of V forms.

In an aërated culture a much more rapid breakdown of glucose occurs than in one not aërated. In order to follow this breakdown, the glucose was estimated by the Hagedorn-Jensen technique in samples removed from an aërated culture at intervals during growth. After 18 hours no glucose could be detected. In spite of the disappearance of the glucose, growth continued and the pH steadily became more alkaline.

The effect of growth in a shallow layer of medium.

In another attempt to increase the growth on lactate under conditions in which the organism grown on glucose remained unaltered, cultures were grown in 100 c.c. conical flasks containing a shallow layer of the ammonia medium (10 c.c.). Growth on glucose or lactate was good but somewhat better on the former; in neither culture was it equal to that in the aërated cultures. Under these conditions the organism grown on glucose remained completely inagglutinable with an O antiserum even after 21 subcultures. The lactate culture as before agglutinated readily with an O antiserum to a titre of 1/3200.

A similar observation was made when an untrained strain of Ty2, *i. e.* one not adapted to use ammonia, was grown in the amino-acid medium with glucose or lactate as the source of energy. Here, however, there seemed to be an even more rapid V to W variation in the lactate cultures and even a development of rough forms. After 12 subcultures a trace of agglutination with normal saline was noticed. On plating out the cultures on nutrient agar, the lactate cultures not only gave rise to colonies of V and W forms as previously described, but also many rough colonies were found. The glucose cultures

showed only V forms. A serum prepared against a rough strain of *Bact. typhi murium* readily agglutinated the organisms grown on the lactate, but not those grown on the glucose medium. The former also absorbed from the serum, agglutinins for the homologous organism. The virulence of the lactate culture was considerably reduced; when a dose of 100×10^6 was injected into 6 mice, all remained alive; an agar-grown control in the same dose killed 50 p.c. With a dose of 200×10^6, 2 out of 6 died; the agar-control killed all 6. The glucose cultures remained fully virulent.

In spite of the loss of O-resistance and virulence, agglutination with a pure Vi antiserum showed no reduction in titre. That this effect was a true Vi agglutination and not a non-specific agglutination of rough organisms was shown by absorption of a pure Vi antiserum with living organisms grown in the lactate medium for 12 subcultures. All Vi agglutinins were removed.

Felix and Pitt (1935) and Kauffmann (1935*a*) found that the Vi antigen may persist even after the organism has become rough. In order to test whether this had occurred, the lactate culture was plated out and a rough colony selected. This was replated twice and then used in the living state for the absorption of a pure Vi antiserum. When it was absorbed with $32{,}000 \times 10^6$, no reduction in titre was shown on testing it against an agar-grown V strain. The rough organism therefore was devoid of Vi antigen.

The foregoing experiments show that, in the lactate culture, in spite of a variation of V forms to W forms and from smooth to rough, organisms possessing Vi antigen are still present after 12 subcultures.

The effect of the age of the culture.

Craigie and Brandon (1936*b*) found that the majority of V strains lost their inagglutinability with an O antiserum if incubated in broth for 18 hours or longer. Four-hour cultures showed uniform absence of O-agglutinability. In order to find out whether the appearance of the O-agglutinable form is dependent on the age of the culture, organisms were grown in the amino-acid-glucose medium for varying periods of time and then tested with a pure O antiserum for agglutination. At the same time the amount of glucose remaining in the culture was estimated by the Hagedorn-Jensen technique. It was found that after 24 hours, when all the glucose in the culture had been exhausted from the medium, O-agglutinable W forms appeared. Similarly a reduction in the concentration of glucose in the medium also led to the appearance of W forms.

The effect of anaërobic conditions of growth on agglutination and virulence.

All the experiments so far described were carried out under aerobic conditions. Several workers (for review, see Wilson, 1930) have shown a relation between virulence and the amount of free oxygen available for growth. In order to test how far the oxygen supply influenced the appearance of W forms in cultures of V forms, cultures were allowed to grow under anaërobic conditions.

Vásárhelyi (1934) found that non-exacting strains of *Bact. typhosum* would not grow anaërobically in a medium containing ammonia as the sole source of nitrogen, and glucose or lactate *plus* nitrate as the hydrogen acceptor, unless tryptophan or asparagine were also added. However, with the Ty2 strain, trained to grow in the ammonia-glucose medium, we have obtained growth,

which can be subcultured indefinitely in the absence of these amino-acids. Growth, however, was poor and considerably delayed. The addition of certain amino-acids (notably cystine) considerably improved the growth, but it was found to be best on the complete amino-acid medium. Since the untrained strain also grew well anaërobically on this medium, there seemed to be no advantage in using the trained strain.

For anaërobic growth, $M/500$ thiolacetic acid (neutralized) was added to the amino-acid medium and the tubes were incubated in McIntosh and Fildes' anaërobic jars, twice evacuated and refilled with hydrogen containing 5 p.c. CO_2. In the lactate cultures, sodium nitrate ($M/40$) was added as the hydrogen acceptor.

When the organism was grown anaërobically in this medium with glucose as the source of energy, and then tested for agglutination against O and Vi antisera, the same result was obtained as in the aërobic culture on this medium, namely, agglutination with Vi antiserum, but no agglutination with O antiserum. When glucose was replaced by lactate *plus* nitrate, a result was obtained which I have been unable to explain. With the usual 18-hour culture used for the agglutination test, there was complete absence of agglutination not only with an O, but also with a Vi antiserum. If the culture were grown for 2 days, however, without opening the jar, the reactions became similar to an aërobic lactate culture, agglutinating both with O and Vi antisera. That this effect was not due to a delayed production of Vi antigen was shown by the complete removal of Vi agglutinins from a pure Vi antiserum by absorption with a living suspension grown for 18 hours. The effect was also shown not to be due to nitrate. Unlike a culture growing under aërobic conditions, a 2-day-old culture in the glucose medium remained inagglutinable with a pure O antiserum. Both cultures remained fully virulent. If the phenomenon of " delay " is excluded, in both aërobic and anaërobic lactate cultures W forms appear. Under anaërobic conditions, however, this variation appears to be much less evident, and there were no signs of the appearance of rough forms even after 19 sub-cultures. Virulence also was not impaired.

The effect of pH.

A difference between glucose and lactate cultures which has not so far been considered is the question of alteration in pH during growth. In a glucose culture, from the beginning of the log phase, there is a steady fall in pH until the glucose has been exhausted or until acid production brings growth to a standstill. If growth continues, the pH starts to rise, owing to the oxidation to CO_2 and H_2O of lactic, formic and other acids produced by glycolysis. As previously mentioned, when a glucose culture was tested at intervals during growth, O-agglutinable forms appeared at the point when the glucose in the medium was exhausted. This is also the point when the reversion in the pH of the medium occurs. When the glucose is replaced by lactate, the pH rises as soon as growth starts and O-agglutinable forms appear directly. There is therefore an apparent relationship between the appearance of O-agglutinable forms and an increase of pH in the medium.

In order to test whether the increase in pH was the cause of the antigenic change, the strain Ty2 was grown in the amino-acid medium with glucose or lactate as the source of energy under the following conditions : (1) Both glucose

and lactate cultures kept constant at pH 7·0. (2) Both cultures kept constant at pH 8·2. (3) The pH of the glucose culture was allowed to fall naturally to pH 6·4, and the lactate cultures adjusted throughout growth to a corresponding pH by the addition of $N/2$ HCl. (4) The pH of the lactate cultures was allowed to rise naturally to pH 8·2, and the glucose cultures adjusted to a corresponding pH throughout the period of growth by the addition of $N/2$ NaOH. Initial cultures were watched continuously for 18 hours and then subcultured under the same conditions. Owing to the impracticability of continuous observation, later subcultures were watched throughout the day and placed in the ice-chest overnight. After 5 subcultures, the cultures were tested for agglutination with an O antiserum. All the glucose cultures, irrespective of pH, were resistant to O agglutination, whilst all the lactate cultures agglutinated to a titre of 1/3200 with an O antiserum. Clearly, the pH has had no influence in the production of O-agglutinable W variants. The appearance of these variants seems to be determined entirely by the nature of the energy source.

The effect of other sources of energy.

In the amino-acid medium, glucose was replaced by various carbon compounds. The agglutination reaction with an O antiserum of organisms grown with different sources of energy was as follows: Growing on galactose, fructose or mannitol the organisms remained inagglutinable with an O antiserum. On glycerol, malate, aspartate, succinate or fumarate or the amino-acid medium without added carbon source, O-agglutinable forms were found.

DISCUSSION.

Explanation of these results is difficult in the absence of any chemical information concerning the Vi antigen. Even an extreme alteration in the process of nitrogen assimilation did not change the antigenic composition or affect the virulence of the organisms. On the other hand, a change in the carbon source from glucose to lactate was at once accompanied by the appearance of W forms which were agglutinated by a pure O antiserum and which had lost their Vi agglutination and virulence. W forms also appeared in glucose cultures under conditions in which growth continued after the glucose had been exhausted; such conditions were—prolonging the period of growth, reducing the amount of glucose in the medium or, especially, increasing the degree of aëration.

Under these conditions, the available sources of carbon and energy after exhaustion of glucose would be the acid breakdown products of glucose, or in the amino-acid medium, the amino-acids themselves. The culture thus resembles one in which lactate or the amino-acids are the sole source of carbon and energy, and these have been shown to be the very conditions leading to a V to W variation. If the organisms were subcultured repeatedly after exhaustion of glucose, then a larger and larger proportion of W to V forms would be present (assuming that a reverse variation of W to V did not occur), and the culture as a whole would become more and more O-agglutinable.

The complete loss of virulence and Vi antigen, associated with prolonged

incubation in an inadequate medium during training to utilize ammonia, may also be attributed to exhaustion of the glucose. Although growth would apparently cease at an early stage, owing to the deficient amount of tryptophan, glycolysis would continue as in the case of any resting suspension. After 6 days' incubation the culture would still contain living organisms, suggesting that a few organisms were still dividing at the end of that time, if only sufficient to slow the normal death-rate. These divisions, when they occurred after the glucose had been removed, would give rise to W forms, and the living organisms of the culture would be gradually replaced by these variants, until in time they would be the only living organisms present. On transference to an adequate medium, the whole culture would then consist of O-agglutinable W forms, and this, in fact, is what was observed. Under anaërobic conditions, provided that the amino-acids did not act to any great degree as hydrogen-acceptors, products resulting from glycolysis would not be utilizable for growth. This must therefore cease after exhaustion of the glucose. Thus the absence of W forms in a 2-days-old culture grown under anaërobic conditions is explained.

These considerations accord with the suggestion of Twort (1907), Lewis (1933) and others that one of the causative factors in variation is the depletion of a more readily utilizable substrate, followed by adaptation to use one less readily used. Similarly the experiments of Craigie and Brandon (1936*b*), in which certain V strains retained their O-inagglutinability in 4-hour cultures, but lost it when incubated for 18 hours or longer, suggest that there was present in the medium (broth) some substance essential for the production of Vi antigen which was rapidly exhausted. Somewhat surprisingly, however, they stated that O resistance was decreased by traces of fermentable sugar.

It is possible that the appearance of O-agglutinability in an aërated culture may be due to alteration in method of glucose breakdown. The organism, growing in a stagnant tube containing glucose, obtains its energy mainly by glycolysis. The effect of aëration would be to change the energy mechanism from one which was predominantly anaërobic (glycolytic) to one which was predominantly aërobic (respiratory). When glucose was replaced by lactate, only an aërobic mechanism would be available, whether the culture was aërated or not. Against this explanation, however, is the fact that glucose cultures contained in flasks in which conditions are mainly aërobic (as shown by the good growth obtained in a parallel lactate culture) show no tendency to become O-agglutinable.

There seems, then, to be a direct relation between the presence of glucose in the medium and the resistance of the organism to agglutination by an O antiserum. It is possible that the organism is able to utilize the glucose but not the lactate molecule for the synthesis of the Vi antigen. This might well occur if the Vi antigen is polysaccharide in nature and comparable with the smooth somatic antigen of the cell. At the same time, however, the lactate culture as a whole contains large numbers of organisms which have retained their Vi antigen, even after several subcultures. Variation must therefore be gradual, and the inability to synthesize the Vi antigen from lactate cannot be absolute.

As Braun (1936) has pointed out, strains of *Bact. typhosum* differ in their ability to synthesize the Vi antigen. It is impossible, however, to make any rigid distinction between them. As previously mentioned, Kauffmann finds

that all freshly isolated strains possess the Vi antigen. Just as *Bact. typhosum* on first isolation requires several amino-acids for growth and can be finally adapted to utilize ammonia, so it is possible that a strain which on first isolation can only synthesize its Vi antigen on ascitic-agar could be adapted to synthesize this from simpler substances. Strain Ty2 may be such a strain. It has been isolated from the body since 1918 and kept on ordinary nutrient agar, but has retained its virulence and Vi antigen. Such an adapted strain would give the appearance of being independent of the medium with regard to the ability to synthesize its Vi antigen, and would be included by Braun in the first group of his scheme. It is only when the medium is still further simplified that changes in the ability to synthesize Vi antigen are revealed.

CONCLUSIONS.

(1) The presence of Vi antigen in strains of *Bact. typhosum* is independent of the source of nitrogen used for growth, even when this is limited to ammonia only.

(2) A relation between the presence of Vi antigen in the cell and the carbon and energy source has been established. Growing in the presence of glucose the organism retains its Vi antigen and is not agglutinated by a pure O antiserum. It remains virulent. The replacement of glucose by lactate or by other 3- or 4-carbon compounds leads to the appearance of O-agglutinable forms which have lost their Vi-antigen.

(3) It is suggested that the glucose molecule is easily utilized by the organism for the synthesis of Vi antigen; the lactate molecule can only be used with difficulty.

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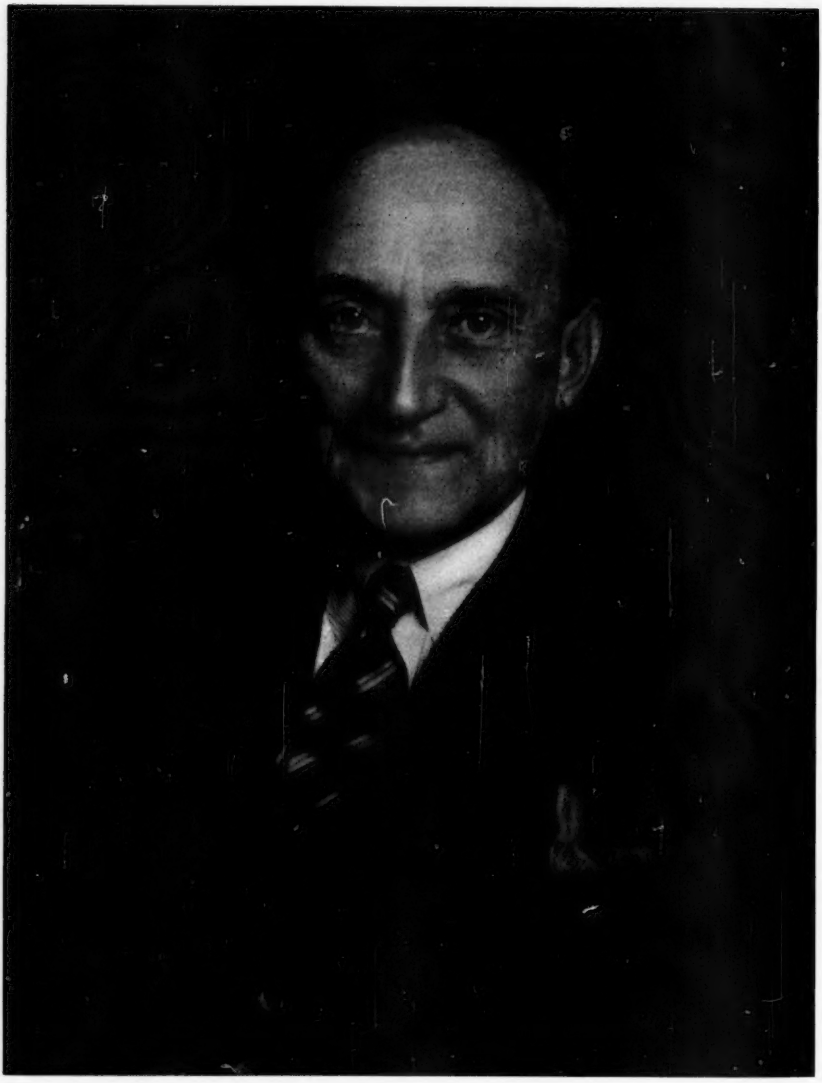

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